MOLECULAR CLONING AND CHARACTERIZATION OF *PACTEROIDES GINGIVALIS* ANTIGENS

BY

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Bacteroides gingivalis, a Gram-negative anaerobic bacterium, is strongly implicated as an etiological agent of periodontal disease. However, its exact role in the disease process has not yet been established. Recombinant DNA technology was applied as an initial approach to a molecular study of *B. gingivalis* antigens by preparing genomic libraries of *B. gingivalis* strain 381 in *E. coli* JM 109 via a pUC 9 plasmid vector. Detection of the expression of *B. gingivalis* antigens was achieved by using *E. coli* adsorbed rabbit anti-*B. gingivalis* sera.

Five different clones were found to stably exhibit *B. gingivalis* antigen expression. Characterization of the antigen-expressing clones demonstrated that clones 2, 5, and 7 agglutinate sheep erythrocytes whereas *E. coli* JM 109 (pUC 9) does not. Clones 5 and 7 were found to have one insert fragment in common and this insert was found to have little or no homology to the insert of clone 2. Clone 5 is also able

to autoagglutinate and it was found that a 760 bp DNA fragment codes for this activity. The common insert of clones 5 and 7 appears to have a *Bacteroides* promoter and to code for the hemagglutinating activity of these clones. The clone 2 insert does not have a *Bacteroides* promoter and is under the control of plasmid *lac* promoter.

Antisera against clones 2, 5, and 7 were found to inhibit the hemagglutinating activity of *B. gingivalis* whereas adsorption of anti-*B. gingivalis* antiserum with clones 2, 5, and 7 partially removed the hemagglutination inhibition activity. However, these clones do not remove the saliva-treated hydroxyapatite (SHA) adherence inhibition activity of anti-*B. gingivalis* antiserum. Western blot analysis of *B. gingivalis* cell lysate antigens using *E. coli* adsorbed antisera against clones 2, 5, and 7 demonstrated that all antisera reacted to 2 major bands of MWs 43,000 and 38,000, which have been reported to be the major bands of the *B. gingivalis* hemagglutinin. *E. coli* adsorbed anti-clones 5 and 7 antisera did not react to the 125,000 protein band expressed in clone 2. In addition, adsorption assays demonstrated that the epitope of the expressed antigen in clone 2 is not related to that of clones 5 and 7. A number of experiments are proposed to further characterize the *B. gingivalis* hemagglutinin genes, the native hemagglutinin molecules, and the significance of hemagglutinins in periodontal disease.

CHAPTER ONE
INTRODUCTION

Periodontal disease (PD) is a chronic inflammatory disease which results in the destruction of the supporting tissues of teeth (Kagan, 1980). Although the specific microbial etiology of PD is not known, it is widely accepted that bacteria are the contributing agents of the disease for the following reasons (Socransky, 1977): 1) disease correlates with the presence of plaque, 2) antibiotics are effective in treatment of PD, and 3) implantation of certain genera of bacteria into gnotobiotic rats results in PD of infected but not of control rats.

Bacteroides gingivalis as the Periodontopathogen

The presence of a complex microflora in the subgingival crevice has complicated the identification of the specific etiologic agents of PD. However, several studies (Socransky, 1977; Slots, 1979; White and Mayrand, 1981) indicate that a few genera, primarily Gram-negative anaerobes, appear to be associated with disease progression. For example, the proportion of Gram-negative anaerobes, especially black-pigmented *Bacteroides,* increases markedly in the subgingival flora with increasing severity of PD. *Bacteroides gingivalis*, previously oral *Bacteroides asaccharolyticus* (Coykendall et al., 1980), is the black-pigmented *Bacteroides* which has emerged as a key putative periodontopathogen for a number of compelling reasons.

B. gingivalis is the predominant bacterial species isolated from periodontal lesions of patients with severe adult periodontitis (Slots,1977; Tanner et al., 1977). Patients with adult periodontitis have been found to have higher levels of IgG antibodies to *B. gingivalis* than normal adults (Mouton et al., 1981) and local immunity to *B. gingivalis* is greater in the more advanced cases than in the early forms of PD (Kagan, 1980). Serum antibody titers to *B. gingivalis* have been reported to decrease after therapy of adult periodontitis patients, suggesting that antibodies to *B. gingivalis* result from infection of this organism (Tolo et al., 1982). *B. gingivalis* is also the most interesting and potentially virulent bacterium cultivable from the subgingival crevice with respect to its capacity for breakdown of tissues and host defense mechanisms (Mayrand and McBride, 1980; Van Steenbergen et al., 1982; Nilsson et al., 1985). In addition, *B. gingivalis* appears to be a causative agent of experimental periodontitis in animals. When *B. gingivalis* is implanted as the monocontaminant in gnotobiotic rats, it causes accelerated alveolar bone loss (Crawford et al., 1977). In a longitudinal study of alveolar bone loss in *Macaca arctoides* (Slots and Hausmann, 1979), the proportion of *B. gingivalis*-type isolates reportedly increased from a minority of the cultivable microbiota prior to bone loss to a majority of the microflora when alveolar bone loss was detectable.

Pathogenicity of *B. gingivalis*

Although *B. gingivalis* has been strongly implicated as an etiological agent of adult periodontitis, its exact role in the disease process has not yet been established. In order to produce PD, it is

likely that bacteria and/or their products may lead to the destruction of the gingival tissues by direct action or indirectly by eliciting an immune response which is detrimental to the host tissues. Periodontopathic bacteria such as *B. gingivalis* must possess characteristics which enable them to colonize the host, survive in the periodontal pocket, possibly invade the gingival tissues, and to destroy the collagenous periodontal ligament, the alveolar bone, and other tissue components surrounding the tooth (Slots and Genco, 1984).

Colonization

It is now recognized that colonization of the oral cavity and many other mucosal environments requires the adherence of bacteria to the surface in order to resist the cleansing action of glandular secretions (Gibbons and Van Houte, 1975; 1980). The adherence of bacteria to host tissues is thus a prerequisite for colonization, which is the initial event in the pathogenesis of disease (Gibbons and Van Houte, 1975). The mechanisms of bacterial adherence involve both ionic and other physical (covalent) forces. Many, if not all pathogenic bacteria possess specific ligands on their surfaces, called "adhesins" which bind to complementary components on host tissues (Gibbons and Van Houte, 1980). The mechanisms of adherence may involve the interaction of carbohydrate binding proteins, or lectins, on bacterial surfaces with carbohydrate-containing receptors on host cells. Binding properties of adhesins may also be facilitated by their hydrophobic domains (Gibbons, 1984).

Components of bacteria which mediate attachment to host tissues include surface structures such as fimbriae, capsular materials, lipopolysaccharides, and membrane-associated extracellular vesicles (Slots and Genco, 1984). In the oral cavity, bacteria can attach to host tissues as well as Gram-positive bacteria in pre-formed plaque (Slots and Gibbons, 1978). The nature of the binding sites on teeth and oral tissues to which Gram-negative bacteria attach has not been well established. In vitro, *B. gingivalis* can attach to and agglutinate erythrocytes (Okuda and Takazoe, 1974; Slots and Gibbons, 1978; Slots and Genco, 1979; Okuda et al., 1981), can adhere in high numbers to human buccal epithelial cells (Slots and Gibbons, 1978; Okuda et al., 1981), crevicular epithelial cells derived from periodontal pockets (Slots and Gibbons, 1978), and surfaces of Gram positive bacteria present in plaque, (Slots and Gibbons, 1978; Schwarz et al., 1987). *B. gingivalis* is also able to adhere to untreated and saliva-treated hydroxyapatite, but in comparatively low numbers (Slots and Gibbons, 1978). *B. gingivalis* has also been reported to bind to HR9 matrix, a material similar to the basement membrane barrier underlying connective tissue (Leong et al., 1985). Recently, it has been reported that *B. gingivalis* can bind fibrinogen and possibly colonize host tissues by attaching to fibrinogen-coated surfaces (Lantz et al., 1986).

Bacterial antagonism may also play an important role in mediating the colonization of *B. gingivalis.* In normal adults, *Streptococcus sanguis* is a predominant organism in supra- and subgingival plaque. *S. sanguis* elaborates sanguicin, a bacteriocin which in vitro inhibits black-pigmented Bacteriodes (Nakamura et al., 1981). Experimental studies in humans have shown that the number of *Streptococcus* species,

including *S. sanguis*, are decreased, while those of *Actinomyces* and black pigmented *Bacteriodes* are increased (Leosche and Syed, 1978) in gingivitis. The mechanism of the proportional decrease of *S. sanguis* is not known but this shift seems to be one of the triggers for the initiation of compositional changes in the subgingival flora. A decrease of sanguicin production may permit the growth of *Actinomyces* species and black pigmented *Bacteriodes* (Takazoe et al., 1984). The growth of *B. gingivalis* may be enhanced by hemin when bleeding occurs in gingivitis, since hemin is a required factor for the cultivation of *B. gingivalis*. Recently, it has been reported that *B. gingivalis* grown under hemin-limited conditions has a reduced virulence in mice compared with bacteria cultured in an excess of hemin (McKee et al., 1986).

When colonization of *B. gingivalis* occurs, there seems to be a change in the bacterial composition in the periodontal pocket. This could be explained by studies of Nakamura et al (1978; 1980) which have demonstrated that *B. gingivalis* produces the black pigment hematin which inhibits the growth of some Gram-positive bacteria, including *S. mutans, S. mitis, A. viscosus, A. naeslundii, A. israelii, Bacterionema matruchotii, Corynebacterium parvum* and *Propionibacterium acnes*. Factors other than inhibitory substances could also affect the colonization of *B. gingivalis*, i.e., the nature of specific antibody and other components in gingival fluid as well as the interactions between the new predominant colonizer and other pre-existing residents (Takazoe et al., 1984).

Evasion of Host Defense

B. gingivalis may survive in the periodontal pocket because it resists phagocytosis. Sundqvist et al. (1982) demonstrated that in vitro, most strains of *B. gingivalis* exhibit a higher resistance to phagocytosis than do less pathogenic strains and that impaired phagocytosis of this bacterial species is related to capsular material. The *Bacteroides* capsule only poorly activates complement, therefore it may function to decrease PMN chemotactic stimulus by masking LPS which strongly activates complement (Okuda et al., 1978). Various experiments have verified that the black pigmented *Bacteroides* strains do not stimulate a strong PMN chemotactic response (Sveen, 1977 a,b; Lindhe and Socransky, 1979; Sundqvist and Johansson, 1980).

Most strains of *B. gingivalis* demonstrate resistance to serum bactericidal systems (Sundqvist and Johansson, 1982). *B. gingivalis* has also been shown to degrade the plasma proteins which are important in the host defense, such as the complement factors C3 and C5 (Sundqvist et al., 1985), immunoglobulins G, A, and M (Kilian, 1981; Sundqvist et al., 1985), alpha-1-proteinase inhibitor, alpha-2-macroglobulin (Carlsson et al., 1984a), haptoglobin, and hemopexin (Carlsson et al., 1984b). It has also been shown that *B. gingivalis* has the capacity to inactivate and degrade the plasma proteins of importance in the initiation and control of the inflammatory response such as C1- inhibitor, antithrombin, and alpha-2-antiplasmin (Nilsson et al., 1985). In addition, *B. gingivalis* can degrade fibrinogen (Lantz et al., 1986) and fibrin (Mayrand and McBride, 1980; Wikstrom et al., 1983); therefore, no effective fibrin barrier is formed around the organism. *B. gingivalis* thus appears to

be an organism fully capable of inactivating the host defense mechanisms against invading bacteria.

Periodontal Tissue Destruction

B. gingivalis possesses a number of components with the potential to destroy gingival tissue constituents as follows: The *B. gingivalis* lipopolysaccharide possesses strong bone resorptive activity (Nair et al., 1982), and inhibits the growth of cultured fibroblasts derived from healthy and periodontally diseased human gingiva (Layman and Diedrich, 1987). The lipopolysaccharide is also a suspected component that stimulates mononuclear cells to produce a factor which strongly stimulates osteoclast-mediated mineral resorption (Bom-Van Noorloos et al., 1986). *B. gingivalis* proteolytic enzymes, especially collagenase (Mayrand and McBride, 1980; Robertson et al., 1982; Mayrand and Grenier, 1985) and a trypsin-like protease (Slots, 1981; Laughon et al., 1982) may be directly involved in periodontal tissue destruction. Enzymes other than proteases may also play an important role in the pathogenesis of periodontal disease. For example, alkaline and acid phosphatases (Slots, 1981; Laughon et al., 1982) may cause alveolar bone breakdown since it has been shown that bacterial phosphatases could cause alveolar bone breakdown (Frank and Voegel, 1978). Bacterial products, i.e., butyrate, propionate (Singer and Buckner, 1981) and volatile sulfur compounds (Tonzetich and McBride, 1981) are also suspected to be toxic to periodontal tissues. Recently, it has been reported that *B. gingivalis* possesses a cartilage-degrading ability which is suspected to be due to its ability to degrade proteinase inhibitors (Klamfeldt, 1986).

It has been suggested that periodontal tissue destruction is mediated not only by bacteria and their products but also by the host defense mechanisms (Horton et al., 1974; Nisengard, 1977). For example, cell mediated immunity has been shown to correlate with the periodontal status of patients (Ivanyi and Lehner, 1970; Patters et al., 1976; Patters et al., 1979). *B. gingivalis* was found to stimulate significantly more lymphoproliferative response in patients with destructive periodontitis than those of normal subjects or those with gingivitis (Patters et al., 1980). A lymphoproliferative response results in the production of lymphokines, several of which can account for some of the destructive effects in periodontal disease. For example, alpha-lymphotoxin can cause cell death and osteoclast activating factor can stimulate osteoclastic bone resorption (Horton et al., 1972). Macrophages have also been suggested to play a role in periodontal tissue destruction. Macrophages may be stimulated by bacterial antigens such as LPS (Wahl, 1982), or by lymphokines (Mooney and Waksman, 1970; Wahl et al., 1975), and subsequently produce tissue-degrading enzymes such as collagenase and other proteases (Wahl et al., 1975; Wahl, 1982). Recently, it has been demonstrated that lipopolysaccharide of *B. gingivalis* can induce circulating mononuclear cells to release collagenase-inducing cytokines. The cytokines then induce collagenase synthesis in human gingival fibroblasts (Health et al., 1987). In addition, IgE, mast cells and basophils may also play a role in periodontal disease (Jayawardene and Goldner, 1977; Olsson-Wennstrom et al., 1978).

Application of Recombinant DNA Techniques to the Study of Periodontal Disease

The recombinant DNA techniques developed during the past few years have proven t be powerful tools for the study of pathogenesis. Several major antigens and virulence factors have been cloned as a means of further characterizing their chemical natures, genetic regulation, and function in various diseases. For example, the cloning and expression of the *Neisseria gonorrhoeae* pilus protein in *E. coli* (Meyer and So, 1982) has helped explain the molecular mechanism of antigenic variation. In other studies, the cloning of several virulence factors including exotoxins (Vodkin and Leppla, 1983; Vasil et al., 1986; Nicosia et al., 1987), enterotoxins (Pearson and Mekalanos, 1982), a hemolysin (Goldberg and Murphy, 1984), and a pneumolysin (Walker et al., 1987) have allowed genetic studies of these proteins and have facilitated the production of safer vaccines. Cloning antigens encoded by unknown genes is made possible by preparing a genomic library in which any gene is theoretically represented. If the number of clones is large enough, it is hoped that any gene can be isolated by screening the library (Perbal, 1984). Genomic libraries of both *Treponema pallidum* (Stamm et al., 1982) and *Legionella pneumophila* (Engleberg et al., 1984 a;b) have been made as a first step in isolating and characterizing their major surface antigens.

The recombinant DNA techniques have, however, been applied only sparingly to the study of Gram-negative anaerobic pathogens and even less to the study of the molecular mechanisms of periodontopathogenesis. The recombinant DNA methodologies offer advantages over previous methods used in the study of oral pathogens. Since several potential

periodontopathogens, including *B. gingivalis*, are difficult to grow to high densities, isolation and purification of antigens, especially those present in small amounts, are often difficult and tedious because of a limited amount of starting material. Cloning specific structures in an organism such as *E. coli* would greatly alleviate these problems since *E. coli* can be grown to high densities easily and cloned structures can be overproduced in *E. coli* (De Franco et al., 1981; Matsumura et al., 1986). This would facilitate the isolation and purification of that structure or component. Also, the cloning and expression of antigens would isolate the antigens at the genetic level. The cloned antigens can then be prepared as products devoid of other *B. gingivalis* antigens. Thirdly, the cloning of *B. gingivalis* antigens would allow a genetic and molecular analysis of the gene(s) which is presently difficult to do due to the lack of a genetic system in *B. gingivalis*. Cloning antigens which may be protective or have potential virulence properties is an, as yet, relatively unexplored approach to define the role of *B. gingivalis* in periodontal disease. It is an approach that may lead to a more complete understanding of the molecular mechanisms of periodontal disease as well as providing molecular tools for the future production of a vaccine for periodontal disease.

The purpose of this study was to employ recombinant DNA techniques to clone antigens of *B. gingivalis* as an initial step in defining their roles in pathogenesis. The specific aims were to

1. Construct genomic libraries (clone banks) of *B. gingivalis* chromosomal DNA in *E. coli*.

2. Identify *E. coli*. transformants which express *B. gingivalis* antigens.

3. Identify cloned antigens which are potential virulence factors.

CHAPTER TWO
CLONING AND EXPRESSION OF *BACTEROIDES GINGIVALIS* ANTIGENS IN *ESCHERICHIA COLI*

Introduction

Several lines of evidence strongly implicate *Bacteroides gingivalis*, a Gram-negative anaerobic bacterium, as an etiological agent of adult periodontal disease (White and Mayrand, 1981; Zambon et al., 1981; Takazoe et al., 1984; Slots and Genco, 1984; Slots et al., 1986). For example, relatively high proportions of *B. gingivalis* have been isolated from adult periodontitis lesions (Slots, 1977; Tanner et al., 1977; Spiegel et al., 1979), patients with adult periodontitis have been found to have higher levels of IgG antibodies to *B. gingivalis* than do normal adults (Mouton et al., 1981; Naito et al., 1984), and local immunity to *B. gingivalis* is greater in the more advanced cases than in the early forms of periodontal disease (Kagan, 1980). *B. gingivalis* also appears to be a causative agent of experimental periodontitis in animals (Crawford et al., 1977; Slots and Hausmann, 1979). In addition, *B. gingivalis* possesses a variety of suspected virulence factors such as proteases, collagenases, immunologlobulin degrading enzymes, and adhesins (Slots and Genco, 1984).

Previous investigations of *Bacteroides* pathogenic mechanisms have employed the isolation and purification of *B. gingivalis* constituents by

B. gingivalis is the predominant bacterial species isolated from periodontal lesions of patients with severe adult periodontitis (Slots,1977; Tanner et al., 1977). Patients with adult periodontitis have been found to have higher levels of IgG antibodies to *B. gingivalis* than normal adults (Mouton et al., 1981) and local immunity to *B. gingivalis* is greater in the more advanced cases than in the early forms of PD (Kagan, 1980). Serum antibody titers to *B. gingivalis* have been reported to decrease after therapy of adult periodontitis patients, suggesting that antibodies to *B. gingivalis* result from infection of this organism (Tolo et al., 1982). *B. gingivalis* is also the most interesting and potentially virulent bacterium cultivable from the subgingival crevice with respect to its capacity for breakdown of tissues and host defense mechanisms (Mayrand and McBride, 1980; Van Steenbergen et al., 1982; Nilsson et al., 1985). In addition, *B. gingivalis* appears to be a causative agent of experimental periodontitis in animals. When *B. gingivalis* is implanted as the monocontaminant in gnotobiotic rats, it causes accelerated alveolar bone loss (Crawford et al., 1977). In a longitudinal study of alveolar bone loss in *Macaca arctoides* (Slots and Hausmann, 1979), the proportion of *B. gingivalis*-type isolates reportedly increased from a minority of the cultivable microbiota prior to bone loss to a majority of the microflora when alveolar bone loss was detectable.

Pathogenicity of *B. gingivalis*

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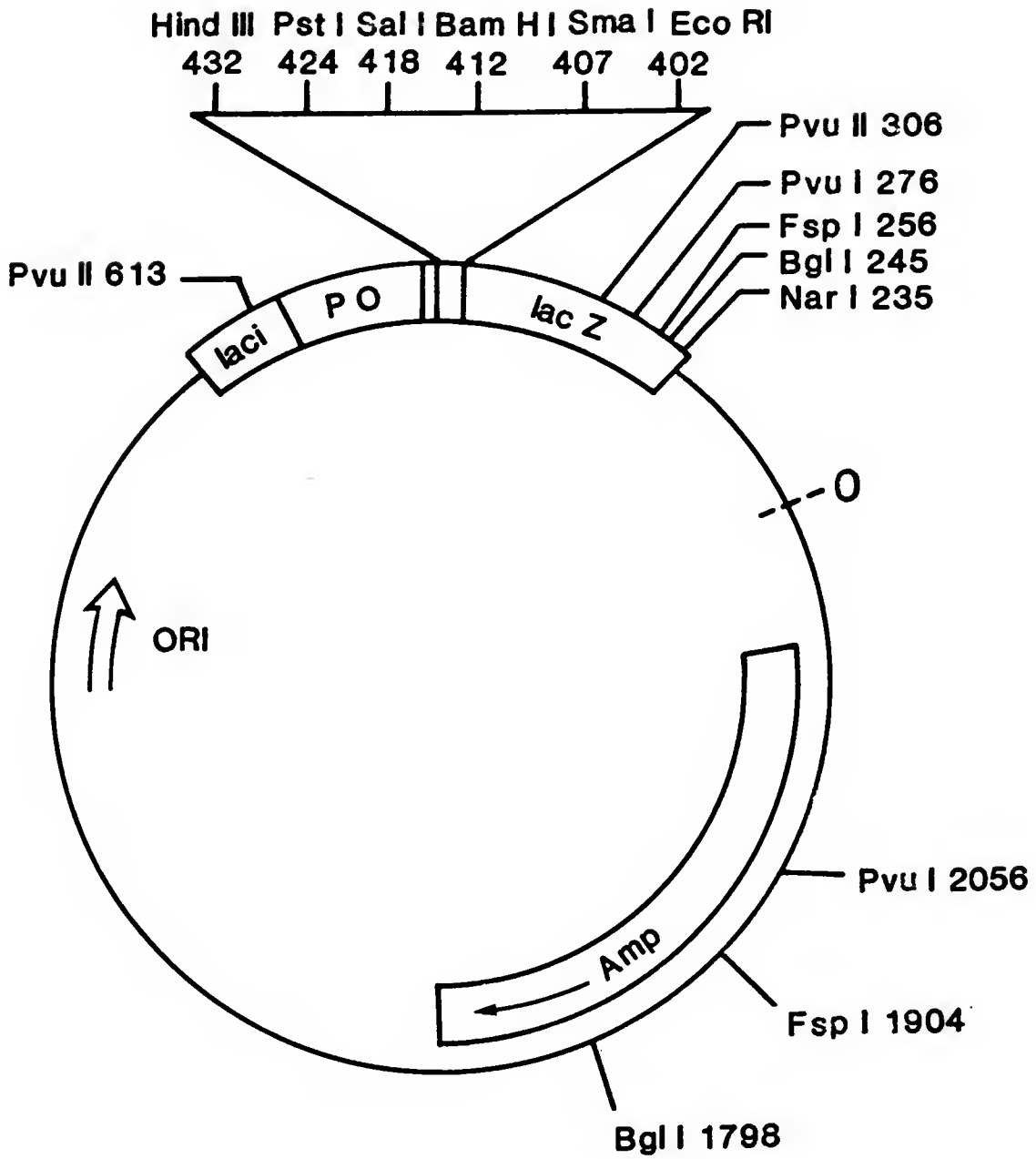
Materials and Methods

Bacterial Strains, Plasmid and Growth Conditions

Bacteroides gingivalis 381 obtained from a stock culture was grown on plates containing Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with sheep blood (5%), hemin (5 micrograms per ml), and menadione (5 micrograms per ml). The organism was also grown in 10 ml of Todd-Hewitt broth (BBL) supplemented with hemin (5 micrograms per ml), menadione (5 micrograms per ml) and glucose (2 milligrams per ml). Cultures were incubated in an anaerobic chamber in a N_2-H_2-CO_2 (85:10:5) atmosphere at 37° C until the log phase of growth was obtained. The 10 ml broth culture was transferred into 25 ml of the same medium and subsequently transferred to 500 ml of medium. Incubation was at 37 °C anaerobically until a late log phase culture was obtained. *E. coli* JM 109 (*rec* Al, *end* Al, *gyr* A96, *thi*, *hsd* R17 sup E44, *rel*Al, Δ(lac-pro AB), [F;*tra* D36, *pro*AB, *lac* IZ M15]) and the plasmid expression vector pUC 9 (Figure 1) were gifts of J. Messing and have been described previously (Vieira and Messing, 1982; Yanisch-Perron et al., 1985). *E. coli* JM 109 was cultured in Luria-Bertani (LB) medium consisting of Bacto-tryptone (10 g per liter), Bacto-yeast extract (5 g per liter), and NaCl (5 g per liter). For solid media, Bacto-agar was added at a final concentration of 15 g per liter. *E. coli* JM 109 transformants were selected and maintained on LB plates containing 50 micrograms of ampicillin per ml.

Figure 1. Map of pUC 9.



pUC 9 (2671 base pairs)

Preparation of Chromosomal DNA from *B. gingivalis*

Chromosomal DNA from *B. gingivalis* 381 was prepared by the method of A. Das (personal communication) as follows: one to three liters of cells were pelleted by centrifugation and washed once with 1x SSC buffer (0.87% NaCl, 0.04% Na citrate) containing 27% sucrose and 10 mM EDTA. The cells were pelleted and resuspended in 1/50 of the original volume of the same buffer at 4°C. Lysozyme (5 mg/ml) in SSC was added to 0.5 mg/ml, the mixture was mixed thoroughly and incubated at 37°C for 10 minutes. Nine volumes of 1x SSC containing 27% sucrose, 10 mM EDTA and 1.11% SDS (prewarmed to 39°C) were added and the cell suspension was incubated at 37° C for 10 to 30 minutes until cell lysis was complete. In order to denature any contaminating proteins, proteinase K was added to a final concentration of 1 mg/ml and the lysate was incubated at 37°C for 4 hours. DNA was extracted twice with phenol, twice with phenol-chloroform (1:1 by volume), and four times with chloroform. Two volumes of absolute alcohol were added and the precipitated DNA was spooled onto a glass rod. The purified DNA was rinsed with 70% ethanol and suspended in TE buffer, pH 8.0 (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

Isolation of Plasmid DNA

Plasmid DNA was isolated by the method of Ish-Horowicz and Burke (1981) in which cells were lysed with SDS-EDTA in the presence of NaOH. Potassium acetate, pH 4.8, was added at 4°C and cell debris, protein, RNA, and chromosomal DNA were removed by centrifugation. The plasmid was precipitated with 2 volumes of ethanol, washed with 70% ethanol, dried, and resuspended in TE buffer at pH 7.5. The plasmid was

separated from contaminating RNA and any remaining chromosomal DNA by cesium chloride density centrifugation in the presence of ethidium bromide. Ethidium bromide and cesium chloride were removed by butanol extraction and dialysis, respectively. The dialyzed plasmid was then phenol- chloroform extracted, ethanol precipitated, and resuspended in TE buffer.

Construction of Genomic Libraries

Purified *B. gingivalis* DNA was partially digested with Sau 3A restriction endonuclease to create fragments of 2-10 kilobases which were ligated to the dephosphorylated Bam HI site of vector pUC 9 with T_4 DNA ligase by standard methods (Maniatis et al., 1982). Genomic fragments were also obtained by partial digestion of the chromosomal DNA with Hind III restriction endonuclease and ligated to the dephosphorylated Hind III site of pUC 9. The recombinant plasmids were used to transform *E. coli* JM 109 by the method of A. Das (personal communication). Briefly, *E. coli* JM 109 was grown to an early log phase (OD_{550} = 0.2) in LB broth. Ten ml of the culture were centrifuged at 5,000 rpm for 5 minutes at 4°C and resuspended in 2 ml of transformation buffer 1 (TFM 1, 10 mM Tris-HCl, pH 7.5, 0.15 M NaCl). The cells were then pelleted and resuspended in 2 ml of TFM 2 (50 mM $CaCl_2$) and incubated on ice for 45 minutes. The cells were again pelleted and gently resuspended in 3 ml of TFM 2, and dispensed into 0.2 ml aliquots. One tenth ml of TFM 3 (10 mM Tris-HCl, pH 7.5, 50 mM $CaCl_2$, 10 mM $MgSO_4$) was added to each aliquot followed by varying amounts of DNA. The cells were then allowed to incubate on ice for 45 minutes, and heat shocked at 37°C for 2 minutes. LB broth (0.5

ml) was added and the cell suspension was incubated at 37° C for 1 hour. Finally, the cells were plated on LB agar containing ampicillin (50 micrograms per ml) and 5-bromo-4-chloro-3-indolyl- β-D-galactopyranoside (X-Gal) (200 micrograms per ml) and incubated for 24 to 48 hours at 37° C. All transformants were stored at -70° C in LB broth with ampicillin (50 micrograms per ml) and 20% glycerol.

Preparation of Antisera

Late exponential phase cells of *B. gingivalis* strain 381 were pelleted, washed with 0.01 M phosphate-buffered saline (PBS) pH 7.2, and resuspended in PBS and 0.01% sodium azide at 4° C for at least 1 hour. The cells were again washed with PBS, resuspended to a concentration of 1 x 10^9 cells per ml and emulsified in an equal volume of Freund's incomplete adjuvant. The cell emulsion was injected in 3 doses at two week intervals for 4 weeks subcutaneously in the back of adult New Zealand rabbits. Each rabbit was given a booster dose 50 to 60 days later. Antisera were collected from the marginal ear veins just prior to immunization and beginning one week after the booster dose. All sera were stored at -20° C.

Rabbit anti-*B. gingivalis* antiserum was adsorbed 4 times with *E. coli* JM 109 harboring pUC 9 plasmid *E. coli* JM 109 (pUC 9) . For each adsorption, *E. coli* cells from 1 liter of a stationary phase culture were washed and mixed with 3 ml of serum at 4° C for 1 hour. The serum was recovered by pelleting the cells at 5,000 x g for 20 minutes. For sonicate adsorption, *E. coli* cells from 500 ml of stationary phase growth suspended in 5 ml of PBS were disrupted by sonication and mixed with *E. coli* cell-adsorbed serum for 1 hour at 4° C. The mixture was centrifuged

at 100,000 x g for 1 hour and the resulting clear serum was stored at -20°C.

Assay of Antibody Titer

Sera were tested for anti-*B. gingivalis* and anti-*E. coli* activities by an enzyme-linked immunosorbent assay (ELISA). *B. gingivalis* cells suspended in carbonate-bicarbonate buffer, pH 9.6, (10^8 cells per well) were fixed to microtiter plates at 4°C overnight. After the wells were washed with 0.5% Tween 20 in PBS, 1% bovine serum albumin (BSA) in PBS was added to each well, and the plates were incubated for 2 hours at room temperature in order to saturate the binding sites. After washing the plates, serially diluted antiserum was added and plates were incubated for 1 hour at room temperature followed by a second wash with 0.5% Tween 20 in PBS. Peroxidase conjugated goat anti-rabbit IgG, diluted 1:1000 in 1% BSA, was added and the plates were again incubated at room temperature for 1 hour. After a final washing, a color-forming substrate solution (O-phenylenediamine, 0.5 g per 100 ml in 0.1 M citrate buffer pH 4.5 and 1.8% hydrogen peroxide) was added, and the plates were incubated for 30 minutes at room temperature. The absorbance at 492 nm was measured with a Titertek Multiscan reader. An absorbance of 0.05 or more over background was considered positive. Background readings were obtained from the wells in which all reagents except anti-*B. gingivalis* antiserum was added. Normal rabbit serum was also tested against *B. gingivalis* antigen.

To test the effectiveness of adsorption, the titers of treated sera were assayed as described above except that *E. coli* JM 109 (pUC9) whole cells were used as the antigen.

Filter-Binding Enzyme Immunoassay

Ampicillin-resistant transformants which formed white colonies in the presence of X-Gal were spotted onto LB agar plates with ampicillin, grown overnight, and blotted onto nitrocellulose filter disks. *B. gingivalis* and *E. coli* JM 109 (pUC 9) were also spotted onto each filter as a positive and negative control, respectively. Duplicate prints of the colonies on nitrocellulose filters were made and colonies on one of each duplicate print were lysed by a 15-min. exposure to chloroform vapor. Filters were then air dried for 30 minutes and soaked for 2 hours in PBS containing 3% bovine serum albumin. After the filters were washed, adsorbed rabbit anti-*B. gingivalis* antiserum was added and the filters were incubated in a solution of peroxidase conjugated goat anti-rabbit immunoglobulin for 1 hour. After washing, the filters were developed in a color-forming substrate solution consisting of 0.06% 4-chloro-1- naphthol and 3% hydrogen peroxide in a 1:4 solution of methanol-TBS (50 mM Tris hydrochloride, 200 mM NaCl, pH 7.4). Clones which developed a blue color were picked and rescreened by the same procedure.

Restriction Analysis of Recombinant Plasmids

Plasmids were isolated from all the clones that were positive in the filter-binding enzyme immunoassay. Restriction endonuclease digestions were performed under conditions described by the manufacturer to produce complete digestion. Agarose gel electrophoresis was performed as described by Maniatis et al. (1982). The size of DNA bands was estimated by comparing the distance of migration to a logrithmic plot of the migration of standard restricted

lambda DNA run on the same gel.

Southern Blot Analysis

Recombinant plasmid and pUC 9 vector DNAs were digested to completion with the appropriate restriction enzymes and run on a 1.2% agarose gel. *B. gingivalis* DNA partially digested with Sau 3A, and Hind III digested *Eikenella corrodens* clone 18 DNA (unpublished) were also loaded in the gel. The DNA was transferred to Biodyne nylon membrane by Southern transfer (Southern, 1975). *B. gingivalis* DNA partially digested with Hind III was nick translated with (α-^{32}P dCTP) (400 Ci/mmol, Amersham Corp., Arlington Heights, Ill.) as described by Maniatis et al. (1982). The membrane-bound DNA was hybridized to the nick-translated probe at 42° C in 50% formamide for 16 hours by the method recommended by the manufacturer (Pall Ultrafine Filtration Corp., Glen Cove, N.Y.) which adapted from Wahl et al. (1979). The membrane was washed at room temperature in wash buffer (2 x SSC and 0.1% SDS) four times each for 5 minutes and twice at 50° C each for 15 minutes in 0.1x SSC, 0.1% SDS. An autoradiogram was obtained with Kodak XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) and Cronex Quanta II intensifying screen (Du Pont Co., Wilmington, Del.).

Assay of the Titer of Anti-*B. gingivalis* Antiserum to *E. coli* Transformants Which Express *B. gingivalis* Antigens

Cultures of each representative clone were prepared by 100 fold dilution of overnight cultures and grown for 2 hours at 37° C. Isopropyl- β-D-thiogalactopyranoside (IPTG) was added to specific

cultures at a final concentration of 1 mM and the cells were pelleted by centrifugation 4 hours later. The cells were washed, resuspended in 1/10 volume of PBS, and the optical density of each suspension was determined at 550 nm. Cell lysate antigen was prepared by breaking the cells with a sonicator. The protein concentration of each lysate was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.). Determination of the titer of anti-*B. gingivalis* 381 against these antigens was performed with the ELISA as described above (10^8 cells or 1 μg protein per well). Normal rabbit serum exhaustively adsorbed with *E. coli* JM109 (pUC9) was also tested in the same manner.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis of the Expressed Antigens

Each of the representative antigen-producing clones was grown to mid-log phase in 3.0 ml of LB broth with 50 micrograms of ampicillin per ml. The cells were pelleted, washed with PBS, resuspended in 0.3 ml of sample buffer (62.5 mM Tris-hydrochloride, 5% 2-mercaptoethanol, 2% SDS, 10% glycerol, 0.002% bromophenol blue, pH 6.8), and boiled for 3 minutes. The *B. gingivalis* cell lysate was mixed with an equal volume of sample buffer and treated in the same manner. SDS-PAGE was performed in a vertical slab gel electrophoresis tank (Hoefer Scientific Instruments, San Francisco, CA.) as described by Laemmli (1970). Samples of 0.03 ml from each clone as well as 40 micrograms of *Bacteroides* cell lysate were run at a constant current of 20 mA per gel through the 4% polyacrylamide stacking gel (pH 6.8) and 30 mA per gel through the 12.5% or 5% separating gel (pH 8.3). The gels were

processed either by staining with Coomassie brilliant blue R2[illegible] (Fairbanks et al., 1971), or used for Western blot analysis.

Western blotting was done as described by Burnette (1981) as follows. Separated antigens on the gel were transferred to nitrocellulose paper (0.45 m) (Schleicher & Schuell Co., Inc., Keene, NH) by electroblotting using the Hoefer apparatus at 60 V overnight with a buffer containing 20 mM Tris base, 150 mM glycine, and 20% methanol (pH 8.3). The blot was visualized as follows. Nitrocellulose sheets were preincubated in a blocking solution of PBS with 2% BSA and 0.1% Tween-20 for 2 hours or overnight. Adsorbed antisera used as probes were usually diluted 1:100 in blocking solution and reacted with the nitrocellulose transfer for 1.5 hours. After washing with distilled water, the membranes were incubated with affinity purified goat anti-rabbit IgG for 1.5 hours. After washing again with distilled water, the membranes were developed in the color-forming substrate solution as described in the filter-binding enzyme immunoassay. The molecular weight of each individual band was estimated by comparison to the molecular weight standard proteins run on the same gel.

Results

Titer of Antisera

Rabbit anti-*B. gingivalis* antiserum had an antibody titer of 1:64,000 to *B. gingivalis* and 1:160 to *E. coli* (pUC 9), whereas normal rabbit serum had an antibody titer of 1:10 to *B. gingivalis* and 1:80 to *E. coli* (pUC 9). Adsorption of anti-*B. gingivalis* antiserum with *E. coli* (pUC 9) resulted in a slight reduction of antibody titer

to *B. gingivalis* and reduced the anti-*E. coli* titer to zero or 1:10.

Identification of *E. coli* Transformants Which Expressed *B. gingivalis* Antigens

Approximately 4,500 tranformants generated from the Sau 3A restricted chromosomal DNA were tested for the expression of *B. gingivalis* antigens by the filter-binding enzyme immunoassay using *E. coli*-adsorbed rabbit anti-*B. gingivalis* serum. Only 1 clone (clone 3) was positive when either lysed or unlysed cells were tested. A total of 1,700 colonies of transformants resulting from Hind III restricted chromosomal DNA were also tested for the expression of *B. gingivalis* antigens. Seven clones gave positive signals. Of these 7 clones, one was positive only when lysed (clone 8) and the rest were positive both when lysed and unlysed (Table 1).

Agarose Gel Electrophoresis of Recombinant Plasmids

To further confirm the positive results of the filter-binding enzyme immunoassay, plasmid DNA was isolated from each positive clone. Electrophoresis of these unrestricted plasmids showed that each clone contained only one recombinant plasmid (Figure 2, lanes 1 through 8).

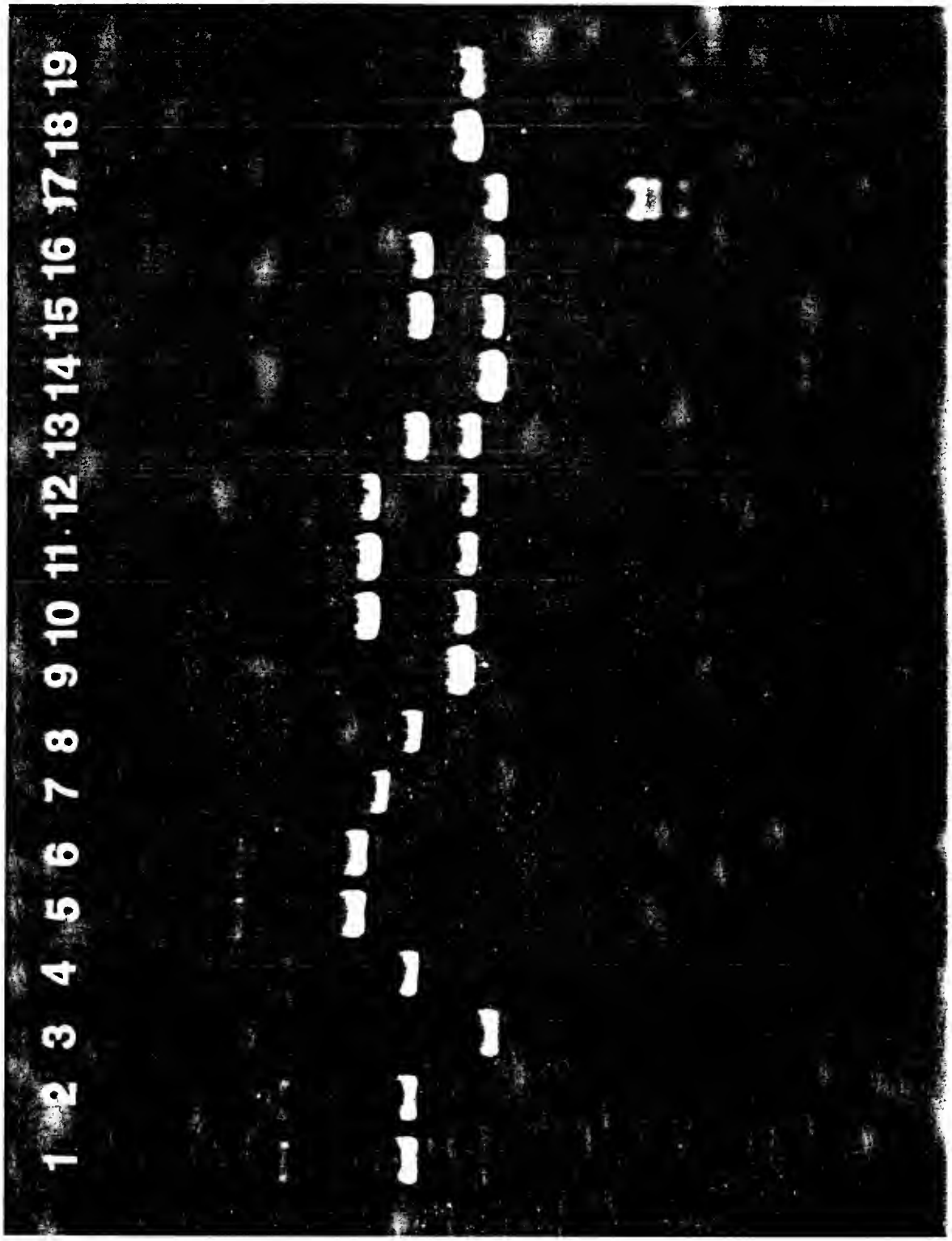
Clone 3, which was constructed by ligation of Sau 3A partially digested *B. gingivalis* DNA with Bam HI cut pUC 9, could not be digested with Bam HI (Figure 3, lane 10). Restriction of pUC 9 with enzyme Sma I and Sal I deletes a 9 bp fragment containing the Bam HI site from pUC 9 (Figure 2, lane 18 and Figure 3, lane 4, see Figure 1 for map of pUC 9). Therefore, clone 3 DNA was restricted with Sma I and Sal I. Restriction analysis revealed a fragment of linear 9 bp-deleted pUC 9

Table 1. Characterization of *E. coli* transformants which express *B. gingivalis* antigens

Clone No.	Colonies reacted with antiserum		Size of *B. gingivalis* DNA cloned (Kb)
	unlysed	lysed	
1 and 2	+[a]	+	3.2
3	+	+	1.1
4	+	+	3.3
5 and 6	+	+	5.5
7	+	+	4.8
8	−[b]	+	3.5

[a] = Positive reaction
[b] = Negative, not reactive

Figure 2. Agarose gel electrophoresis of recombinant plasmids.
Lanes: 1 - 8, undigested recombinant plasmids from clones 1 - 8; 9, pUC 9 digested with Hind III; 10 - 13, recombinant plasmids from clones 5, 6, 7, and 8 digested with Hind III; 14, pUC 9 digested with Pvu II; 15 - 17, recombinant plasmids of clones 1, 2, and 4 digested with Pvu II; 18, pUC 9 digested with Sma I and Sal I; 19, recombinant plasmid of clone 3 digested with Sma I and Sal I.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

and 2 fragments of insert (Figure 2, lane 19 and Figure 3, lane 5). Restriction analysis with different enzymes (Figure 3) showed that the size of insert of clone 3 was approximately 1.1 kb.

Clones 1, 2, 4, 5, 7, and 8 were generated from Hind III-restricted chromosomal DNA. After digestion with Hind III, only clones 5, 6, 7, and 8 revealed fragments of the linear pUC 9 vector and fragments of *B. gingivalis* DNA inserts (Figure 2, lanes 10 through 13). Plasmid DNAs of these clones were restricted with various enzymes and analyzed by gel electrophoresis (Figure 4). The estimated size of inserts of clones 5, 6, 7, and 8 are 5.5, 5.5, 4.8, and 3.5 kb, respectively (Table 1). Thus clones 5 and 6 were found to contain plasmids of the same size and identical restriction fragments.

Although clones 1, 2, and 4 were generated from Hind III restricted DNA, they did not result in fragments of linear pUC 9 after Hind III digestion (Figure 5, lanes 6, 11, and 16). These cloned DNAs were then restricted with Pvu II, which generates a 307 bp fragment containing the polylinker-cloning sites from pUC 9 (Figure 1 and Figure 2, lane 14 and Figure 5, lane 4). Clones 1, 2, and 4 revealed fragments of linear 307 bp-deleted pUC 9 and inserts associated with the deleted fragment (Figure 2, lanes 15, 16, and 17). These cloned DNAs were digested with various restriction enzymes and analyzed by agarose gel electrophoresis (Figure 4). The size of inserts of clones 1, 2, and 4 were estimated to be 3.2, 3.2, and 3.3 kb, respectively (Table 1). Clones 1 and 2 also contained plasmids of the same size and identical restriction fragments.

Figure 3. Agarose gel electrophoresis of different restriction digests of the recombinant plasmid from clone 3.
Lanes: 1, DNA marker-Hind III/Eco RI digest of lambda DNA; 2, undigested pUC 9; 3, pUC 9 digested with Hind III; 4, pUC 9 digested with Sma I and Sal I; 5, 6, 7, 8, 9, and 10, recombinant plasmid from clone 3 digested with Sma I and Sal I, Sma I alone, Sal I alone, Hind III, Eco RI, and Bam HI, respectively; 11, undigested recombinant plasmid from clone 3; 12, DNA marker-Hind III digest of lambda DNA.

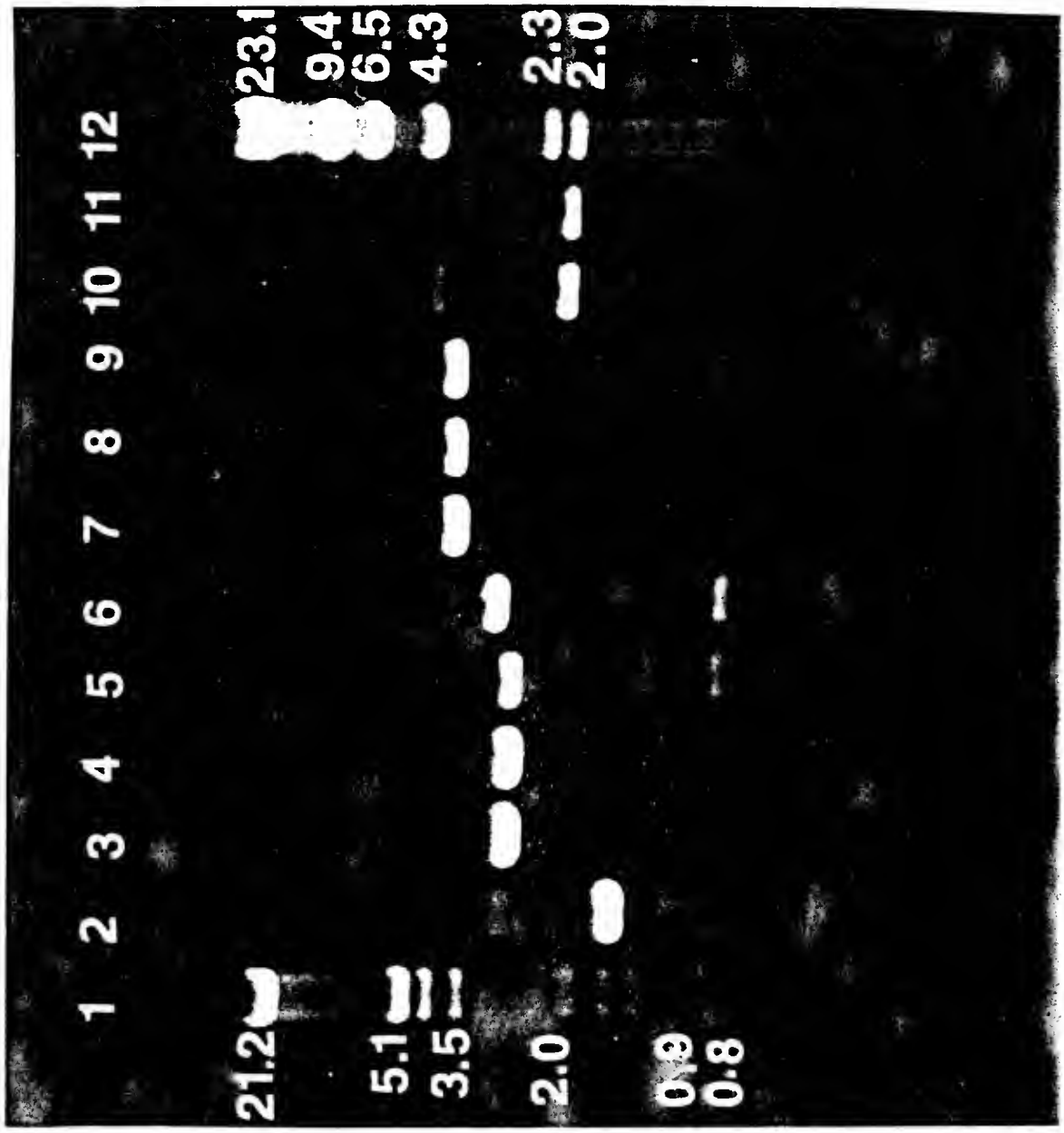

1 2 3 4 5 6 7 8 9 10 11 12
21.2
5.1
3.5
2.0
0.9
0.8
23.1
9.4
6.5
4.3
2.3
2.0

Figure 4. Agarose gel electrophoresis of different restriction digests of recombinant plasmids from clones 5, 6, 7, and 8.
Lanes: 1, DNA marker-Hind III/Eco RI digest of lambda DNA; 2, pUC 9 digested with Hind III; 3, 4, and 5 recombinant plasmid from clone 5 digested with Hind III, Eco RI and Bam HI respectively; 6, 7, and 8, recombinant plasmid from clone 6 digested with Hind III, Eco RI and Bam HI, respectively; 9, 10, and 11, recombinant plasmid from clone 7 digested with Hind III, Eco RI and Bam HI, respectively; 12, 13, and 14, recombinant plasmid from clone 8 digested with Hind III, Eco RI and Bam HI, respectively; 15 - 18, undigested recombinant plasmids from clones 5 - 8; 19, DNA marker-Hind III digest of lambda DNA.

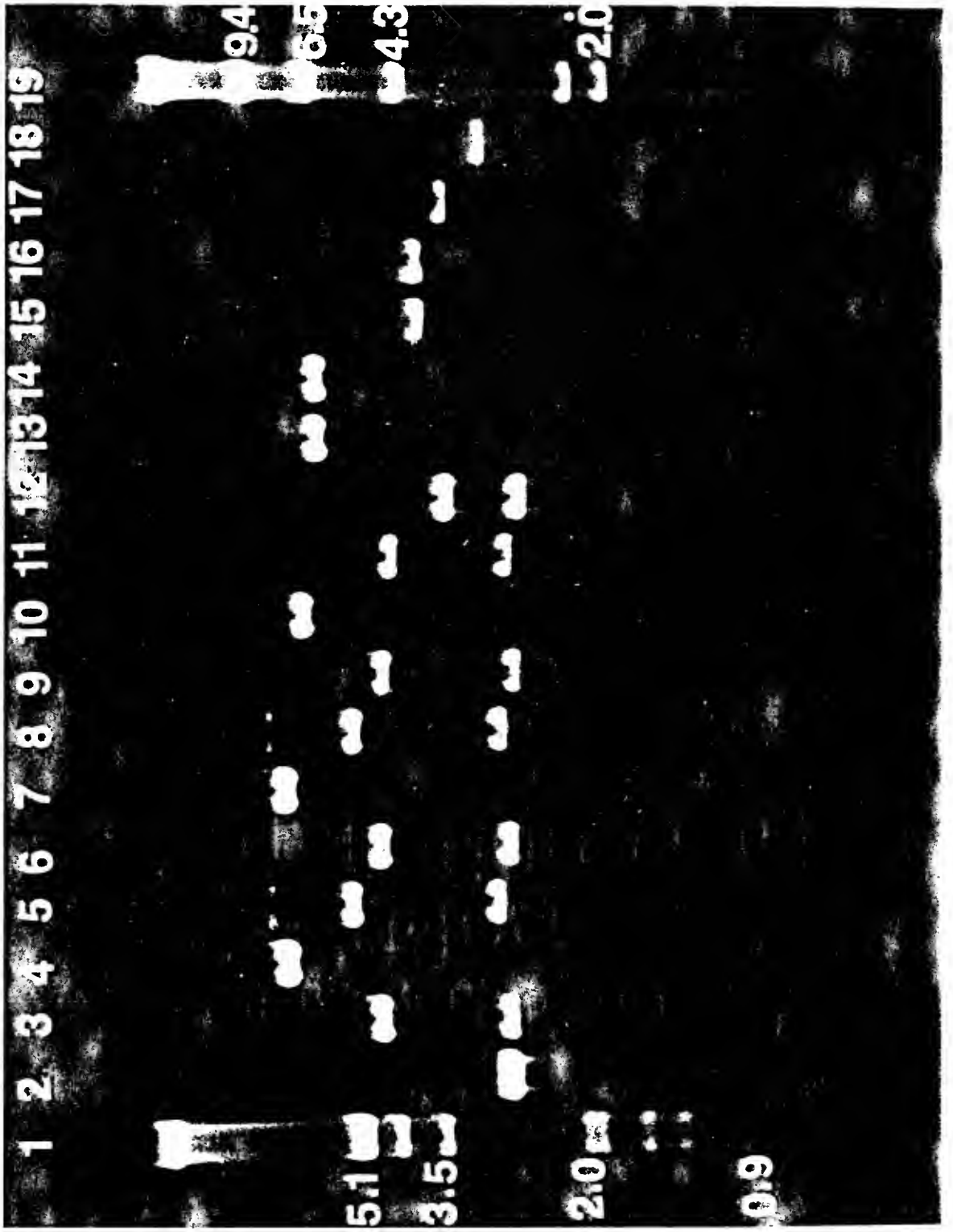
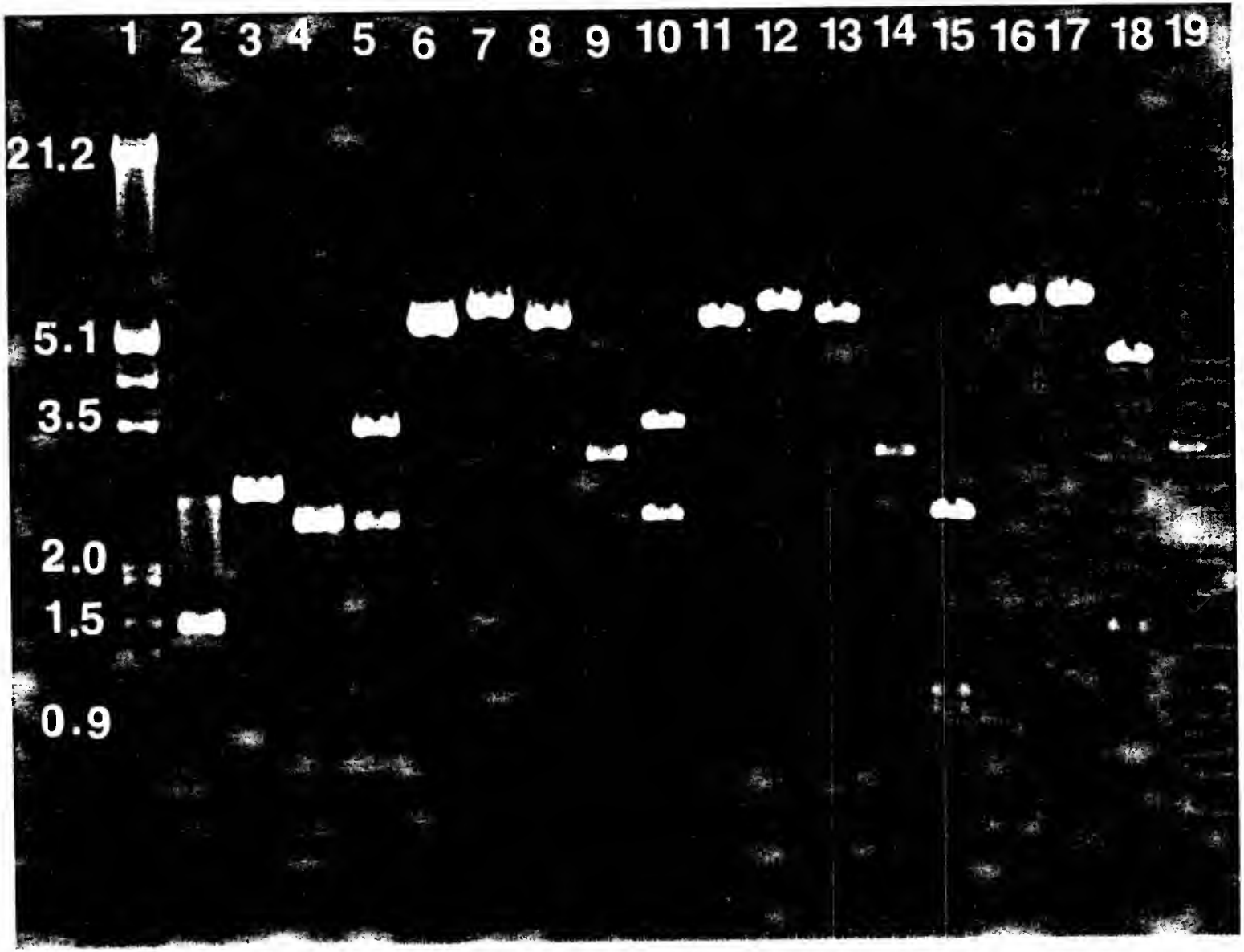

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
9.4
6.5
4.3
2.0
5.1
3.5
2.0
0.9

Figure 5. Agarose gel electrophoresis of different restriction digests of recombinant plasmids from clones 1, 2, and 4.
Lanes: 1, DNA marker-Hind III/Eco RI digest of lambda DNA; 2, pUC 9 undigested; 3, pUC 9 digested with Hind III; 4, pUC 9 digested with Pvu II; 5, 6, 7, and 8, recombinant plasmid from clone 1 digested with Pvu II, Hind III, Eco RI and Bam HI, respectively; 9, undigested recombinant plasmid from clone 1; 10, 11, 12, and 13, recombinant plasmid from clone 2 digested with Pvu II, Hind III, Eco RI, and Bam HI, respectively; 14, undigested recombinant plasmid from clone 2; 15, 16, 17, and 18, recombinant plasmid from clone 4 digested with Pvu II, Hind III, Eco RI, and Bam HI, respectively; 19, undigested recombinant plasmid of clone 4.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
21.2
5.1
3.5
2.0
1.5
0.9

Hybridization of Recombinant Plasmids with *B. gingivalis* DNA Probe

Southern blot analysis was also performed to confirm that the DNA inserts were derived from the *B. gingivalis* DNA. As can be seen in Figure 6, the hybridization pattern of most of the insert fragments showed dark bands of homology to the *B. gingivalis* chromosomal DNA probe. The pUC 9 showed a faint band with homology to the probe. Increasing the stringency of the wash (65° C for 1 hour) did not significantly change the hybridization pattern. However, a shorter exposure of the autoradiograph eliminated the background of pUC 9 but the two smallest insert bands from clone 4 also disappeared. The control DNA from *Eikenella corrodens* did not hybridize with the *B. gingivalis* DNA probe (Figure 6, lane 12).

Titer of Anti-*B. gingivalis* Antiserum to *E. coli* Transformants

Anti-*B. gingivalis* antiserum was able to detect antigen expression in all positive clones except clone 8 in an enzyme-linked immunosorbent assay (ELISA) (Table 2). The antiserum reacted with both whole cell and cell lysate antigens. Isopropyl- β-D-thiogalactopyranoside (IPTG) was not necesary to induce antigen expression. However, in the presence of IPTG, clones 2 and 3 showed higher antigen expression, especially when the cell lysate preparations were tested.

Determination of the Expressed Antigens in *E. coli* JM 109

Five stable representative clones were analyzed for antigen expression by SDS-PAGE and Western blot analysis. As can be seen in Figure 7, only clones 2 and 3 produced antigens detectable by *E. coli* adsorbed anti-*B. gingivalis* antiserum in the Western blot. Antigens

Figure 6. Hybridization of recombinant plasmids with ^{32}P labeled *B. gingivalis* DNA probe.
A. Agarose gel electrophoresis of DNA before Southern transfer.
Lanes: 1, Sau 3A partially digested *B. gingivalis* DNA; 2, pUC 9 digested with Pvu II; 3, recombinant plasmid from clone 1 digested with Pvu II; 4, recombinant plasmid from clone 2 digested with Pvu II; 5, recombinant plasmid from clone 4 digested with Pvu II; 6, pUC 9 digested with Hind III; 7, recombinant plasmid from clone 5 digested with Hind III; 8, recombinant plasmid from clone 6 digested with Hind III; 9, recombinant plasmid from clone 7 digested with Hind III; 10, recombinant plasmid from clone 3 digested with Sma I and Sal I; 11 and 12, recombinant plasmid from *Eikenella corrodens* clone 18 digested with Hind III.
B. Autoradiograph of Southern blot hybridization of the agarose gel in panel A.

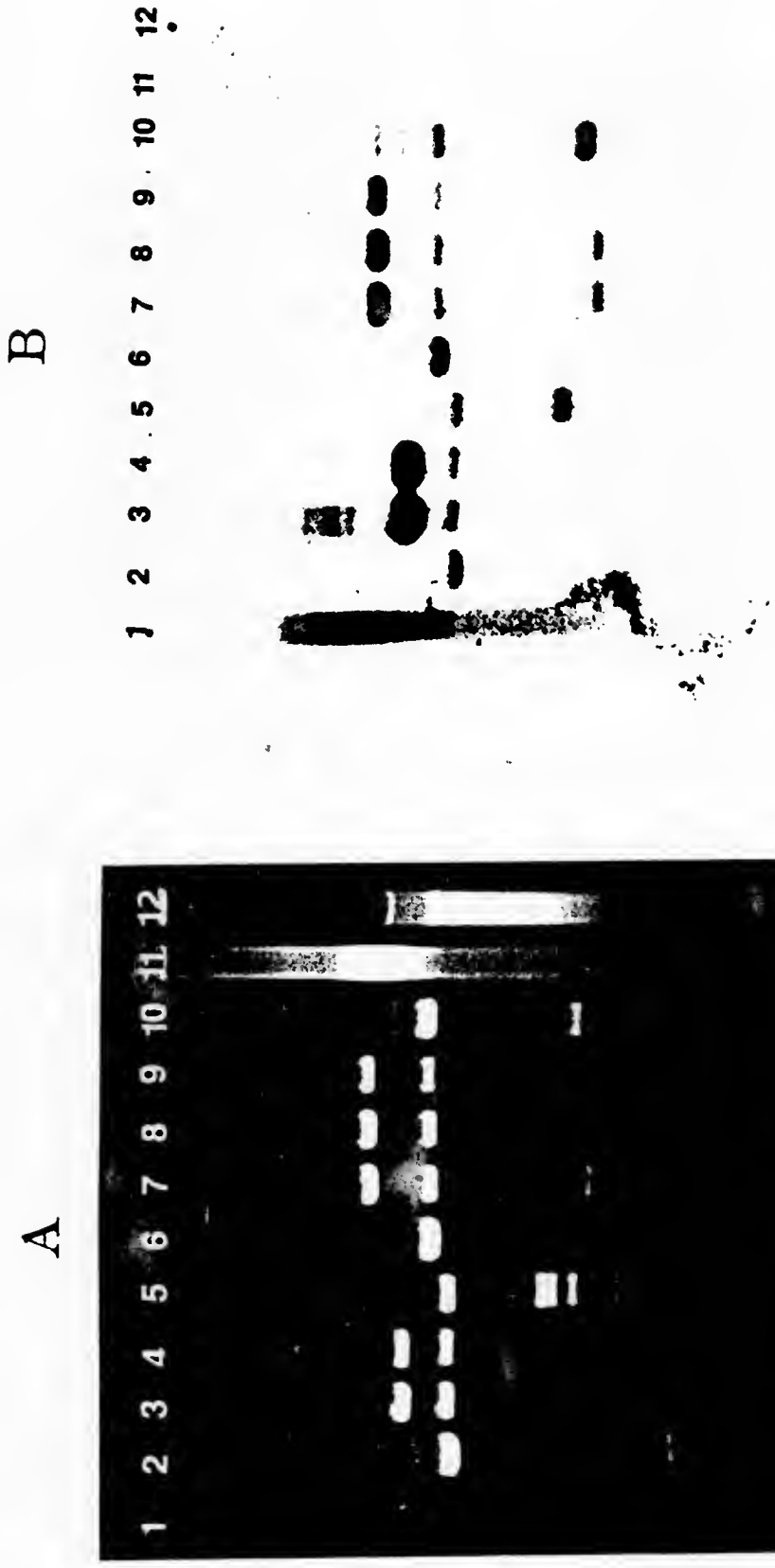

B
1 2 3 4 5 6 7 8 9 10 11 12
A
1 2 3 4 5 6 7 8 9 10 11 12

Table 2. Titer of anti-*B. gingivalis* antiserum against *E. coli* transformants which express *B.gingivalis* antigens

Organism	Antibody titers[a] against test antigens[b]			
	Whole cell		Cell lysate	
	IPTG⁻	IPTG⁺	IPTG⁻	IPTG⁺
Clone 1	320	NT[c]	320 - 640	NT
2	320	640	320 - 640	1,280 - 2,560
3	20	160	40 - 160	1,280
4	20 - 100	20 - 40	20 - 40	20 - 40
5	40 - 80	40 - 80	40 - 80	40 - 80
6	40	NT	40	NT
7	40	40	40	40
8	0	0	0	NT
E. coli JM 109 (pUC 9)	0 - 10	0 - 10	0 - 10	0 - 10
B. gingivalis	40,960 - 64,000	NT	NT	NT
Control NRS[d]				

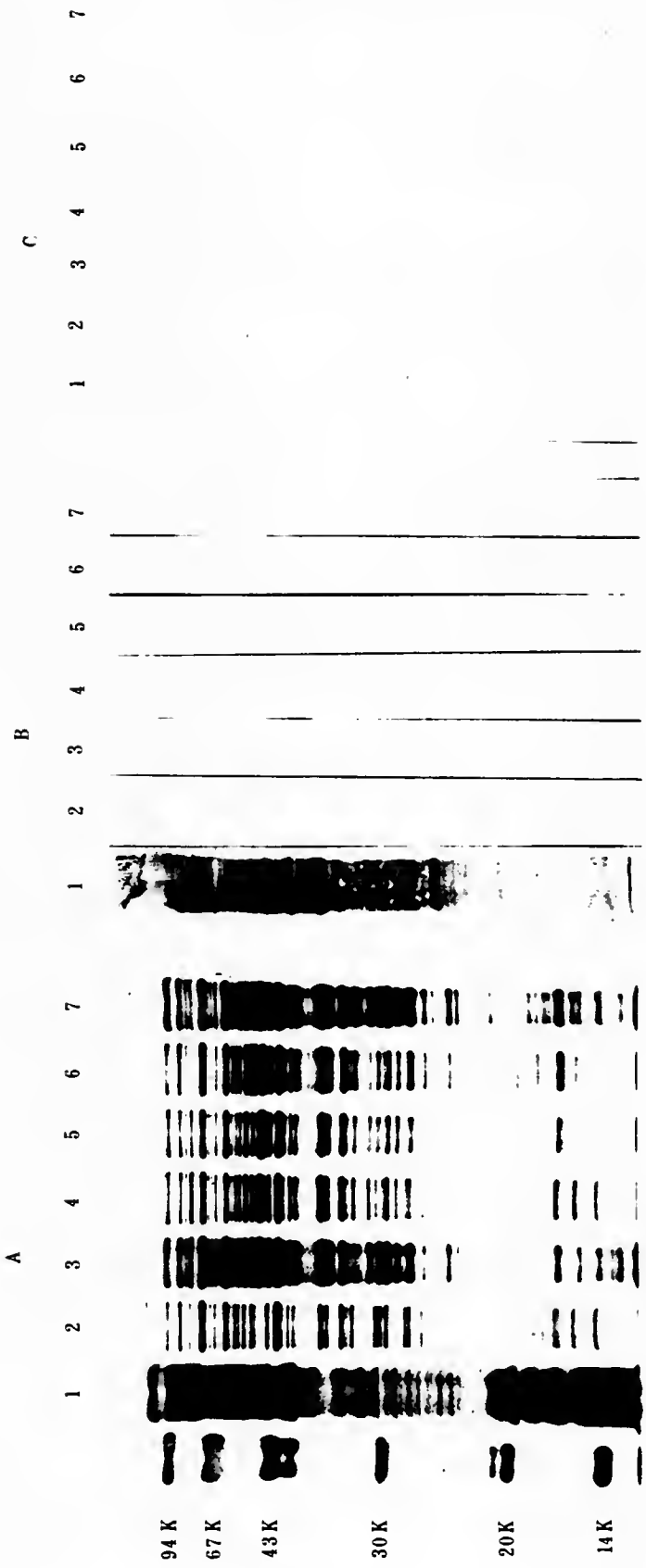
[a] Number designates the reciprocal dilution of the sera which gave OD_{492} reading of 0.05 or more over the background. Antiserum was exhaustively adsorbed with *E. coli* JM109 (pUC 9).

[b] Antigens were prepared from cultures grown without IPTG (IPTG⁻) or in the presence of IPTG (IPTG⁺).

[c] Not tested.

[d] Normal rabbit serum exhaustively adsorbed with *E. coli* JM 109 (pUC 9) did not react to test antigens.

Figure 7. SDS-PAGE on 12.5% acrylamide and Western blot analysis of expressed *B. gingivalis* antigens. Molecular weight standards (Pharmacia Fine Chemicals, Piscataway, N.Y.) are phosphorylase b (94 K, 94,000 molecular weight), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and α-lactalbumin (14,400).
Lanes: 1, *B. gingivalis* cell lysate (40 μg); 2 to 7, whole cell samples of clones 2, 3, 4, 5, 7, and *E. coli* JM 109 (pUC 9) as described in the methods.
A. The gel after Coomassie blue stain. B. The blot was probed with *E. coli*-adsorbed anti-*B. gingivalis* antiserum. C. The blot was probed with normal rabbit serum.

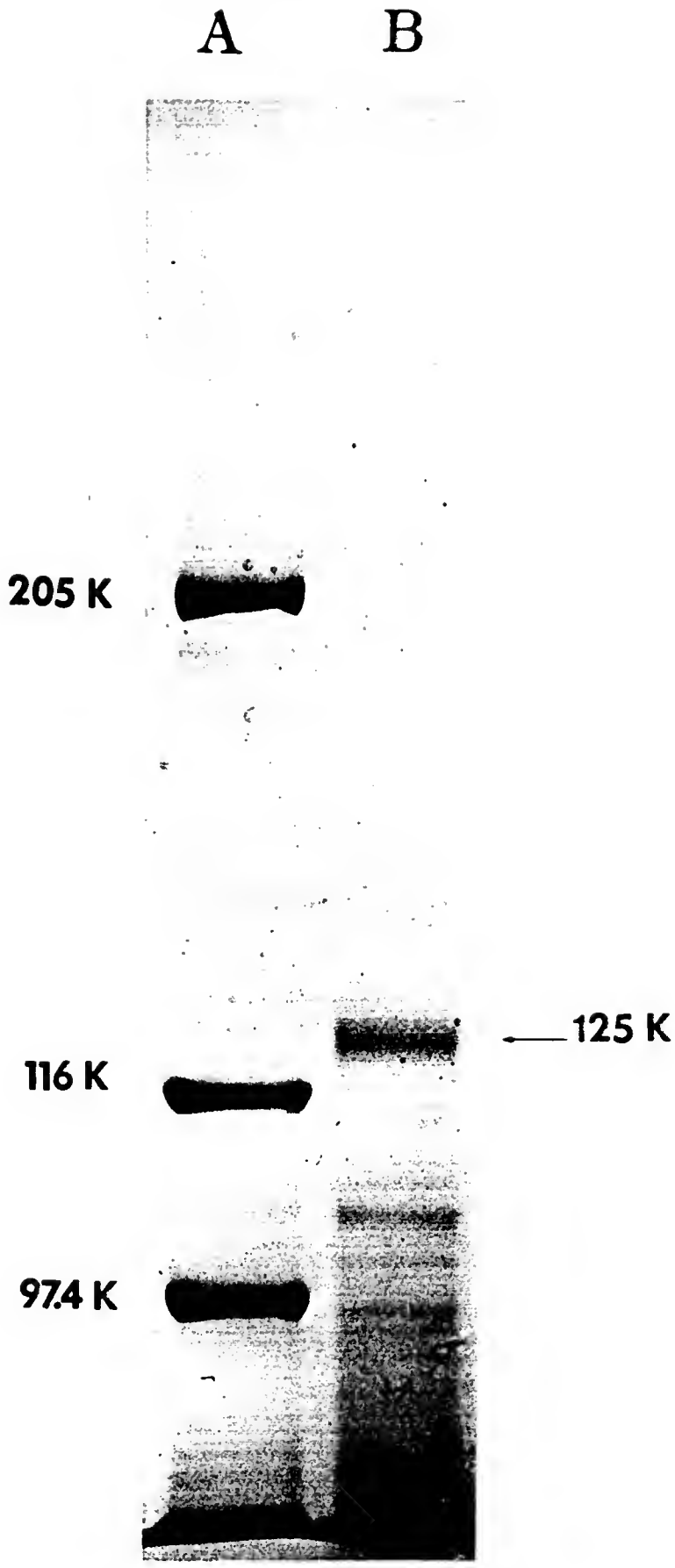

C
1 2 3 4 5 6 7
B
1 2 3 4 5 6 7
A
1 2 3 4 5 6 7
94 K
67 K
43 K
30 K
20 K
14 K

expressed in clones 4, 5, and 7 were not detected by Western blot analysis. Normal rabbit serum reacted to some common antigens among these clones and *E. coli* JM 109 (pUC 9). The anti-*B. gingivalis* antiserum did, however, react with a protein band of approximately 140,000 (140 K), as well as a smear of lower molecular weight from clone 2. Multiple bands of 30 to 50 K from clone 3 were also detected. These particular polypeptides were not detectable in *E. coli* JM 109 (pUC 9) preparations (Figure 7, lane 7). A whole cell preparation from clone 2 was also separated in a 5% SDS polyacrylamide gel and the expressed protein was estimated to have a molecular weight of 125 K (Figure 8).

Discussion

Genomic libraries of *B. gingivalis* DNA were constructed in the plasmid expression vector pUC 9, which contains the pBR 322 origin of replication, the pBR 322 ampicillin resistance gene, and a portion of the *lac* Z gene of *E. coli* which codes for the α-peptide of β-galactosidase (Figure 1). The amino terminus of the *lac* Z gene contains a polylinker region which has multiple unique cloning sites. Transformation of *E. coli* JM 109, which is defective in β-galactosidase, with pUC 9 complements the bacterial β-galactosidase activity, resulting in the ability of the bacterial cell to metabolize the lactose analog X-Gal to a blue color. Cloned DNA inserted in the polylinker region will interrupt the *lac* Z gene of the plasmid. Therefore *E. coli* transformants resulting from recombinant plasmids will be unable to metabolize X-Gal and appear as white colonies on X-Gal containing plates. The advantages to this plasmid are 1) DNA inserted

Figure 8. SDS-PAGE (on 5% acrylamide) of expressed *B. gingivalis* antigen in clone 2.
Lanes: A) Molecular weight standards (Sigma Chemical Co., St. Louis, Mo.) are myosin (205 K, 205,000), β-galactosidase (116,000), phosphorylase B (97,400); B) Whole cell sample of clone 2.


A
B
205 K
125 K
116 K
97.4 K

into any of the cloning sites, which are downstream from a strong promoter, should be expressed whether or not a *B. gingivalis* promoter is cloned with a structural gene, 2) transformants containing a recombinant plasmid are easily detected upon initial selection, and 3) the multiple cloning sites make it a versatile cloning vector which is especially useful for subcloning.

Five different *E. coli* clones stably exhibited *B. gingivalis* antigen expression. These antigens were detected in intact cells both by filter-binding enzyme immunoassay (Table 1) and ELISA (Table 2). Although it has not yet been confirmed by immunoelectronmicroscopy, it is likely that these *Bacteroides* antigens are located on the *E. coli* cell surface, and therefore must contain a leader peptide in order to be translocated to the *E. coli* surface (Oliver, 1985). This result suggests that *B. gingivalis* surface antigens can be processed as well as expressed in *E. coli.*

Clones 1 and 2 have undergone some kind of DNA rearrangement, i.e., the recombinant plasmids, when cut by Hind III, did not result pUC 9 and insert band but showed one large band and one small band (Figure 2, lanes 10 and 11). This apparent rearrangement may result from a deletion at one Hind III end of the insert and another Hind III end may still be intact.

Clone 2 was found to encode a polypeptide with an average molecular weight of 125 K, seen in polyacrylamide gels and detected by Western blot analysis (Figures 7 and 8). The smear at the lower molecular weight seen in the blot may be the degraded product of this expressed antigen, since *E. coli* has a functional *lon* gene which encodes for the enzyme involved in degradation of internal abnormal

proteins (Charette et al., 1981; Chung and Goldberg, 1981; Waxman and Goldberg, 1982).

The function of the *lac* promoter in pUC 9 does not depend on IPTG; it is, however, enhanced by IPTG, since *E. coli* JM 109 (pUC 9) grows as blue colonies on medium containing X-Gal in the absence of IPTG. Expression of the *B. gingivalis* antigen in clone 2 occurs either in the presence or absence of IPTG but is enhanced by IPTG stimulation. This result suggests that the direction of transcription of this DNA insert is the same as that of β-galactosidase and is likely to be under the control of the *lac* promoter. Assuming an average molecular weight of 100-125 for an amino acid, the insert of clone 2, estimated to be 3,200 bp, could encode for a 125 K polypeptide. The expressed polypeptide may be fused to the major portion of the α-peptide of β-galactosidase which would add approximately 100 amino acids to the expressed polypeptide. The expression of the clone 3 antigen was also found to be stimulated by IPTG in the same manner as clone 2. The size of the clone 3 insert (1,100 bp) is large enough to encode for the expressed antigen (30 to 50 K) observed by Western blotting.

The synthesis of *Bacteroides* antigens in clones 4, 5, and 7 was not found to depend on the presence of IPTG or to be enhanced by IPTG (Table 2). This suggests that a functional *Bacteroides* promoter is included with the structural gene of each clone. However, antigen expression of these clones cannot be detected by Western blot analysis. This might be due to 1) the antigens not being transferred to the nitrocellulose sheets, 2) the transferred antigens containing altered conformations which are not recognized by the antiserum, or 3) the antigen expression being too low to be detected.

These results have demonstrated that the *B. gingivalis* genome can be cloned and expressed in *E. coli.* The cloned antigens are presently being identified and further characterized for functional properties. The cloning of *B. gingivalis* genes is an approach that provides new tools for investigations into the pathogenecity of *B. gingivalis.*

CHAPTER THREE
CHARACTERIZATION OF *BACTEROIDES GINGIVALIS* ANTIGENS SYNTHESIZED IN *ESCHERICHIA COLI*

Introduction

Bacteroides gingivalis possesses several potential virulence factors which may 1) promote its colonization in the host, 2) resist host defenses, and 3) cause destruction of periodontal tissues (Slots and Genco, 1984). Colonization, the initial event in the establishment of disease, requires the adherence of bacteria to host tissues (Gibbons and Van Houte, 1975), therefore bacterial surface components which mediate bacterial adherence are considered to be important virulence factors. In the oral cavity, bacteria can attach to host tissues as well as to bacteria in pre-formed plaque (Slots and Gibbons, 1978). The nature of the binding sites on teeth and oral tissues to which periodontopathic bacteria, including *B. gingivalis*, attach has not been well established. In vitro, *B. gingivalis* can attach to and agglutinate erythrocytes (Okuda and Takazoe, 1974; Slots and Gibbons, 1978; Slots and Genco, 1979; Okuda et al., 1981), can adhere in high numbers to human buccal epithelial cells (Slots and Gibbons, 1978; Okuda et al., 1981), to crevicular epithelial cells derived from periodontal pockets (Slots and Gibbons, 1978), and to surfaces of Gram positive bacteria present in plaque, (Slots and Gibbons, 1978; Schwarz et al., 1987). In addition it will adhere to untreated and saliva-treated hydroxyapatite (SHA), but in comparatively low numbers (Slots and Gibbons, 1978). *B. gingivalis* has also been reported to bind to

HR9 matrix, a material similar to the basement membrane barrier underlying connective tissue (Leong et al., 1985). Recently, it has been reported that *B. gingivalis* can bind to fibrinogen and possibly colonize host tissue by attaching to fibrinogen-coated surfaces (Lantz et al., 1986).

Since the components involved in *B. gingivalis* adherence *in vivo* are, at present, ill defined, the expression of any structure detected by *in vitro* methods thus needs to be examined. Therefore, the antigen-expressing clones described in Chapter Two were tested for the expression of adhesins for saliva-treated hydroxyapatite (SHA adhesin) and erythrocytes (hemagglutinin). This chapter describes the assay for the SHA adhesin by testing for removal of SHA adherence inhibition by anti-*B. gingivalis* antiserum and the assay for hemagglutinin by a direct hemagglutination test. The clones which were able to agglutinate erythrocytes were analyzed by restriction analysis of their *B. gingivalis* DNA inserts and DNA homologies were tested by Southern blot hybridization. Antibodies against these clones were made in rabbits and used as probes to identify the native antigens of *B. gingivalis* by Western blot hybridization. *B. gingivalis* DNA inserts from clones 2 and 7 were used as probes in the hydridization of several restricted *B. gingivalis* chromosomal DNAs to determine whether these inserts are adjacent to each other in the chromosomal DNA.

Materials and Methods

Bacterial Strains and Growth Conditions

Bacteroides gingivalis 381 was cultured in Todd-Hewitt broth as described in Chapter Two. *E. coli* transformants were cultured in LB medium containing 50 micrograms of ampicillin per ml by preparing 100 fold dilutions of overnight cultures followed by incubation for 2 hours at 37° C. IPTG was added to the cultures, when used at a final concentration of 1 mM, and the cultures were incubated for an additional 4 hours.

Assay for Removal of SHA Adherence Inhibition by Anti-*B. gingivalis* Antiserum

Aliquots of anti-*B. gingivalis* antiserum were adsorbed with each antigen-expressing clone as well as *E. coli* JM 109 (pUC 9) as described in Chapter Two. The titer of each adsorbed antiserum was tested against ea h clone and *B. gingivalis* whole cell antigen by ELISA as described above.

Whole paraffin-stimulated human saliva was collected and heated at 56° C for 30 minutes to inactivate degradative enzymes. Extraneous debris and cells were removed by centrifugation at 12,000 rpm for 10 minutes and sodium azide was added to a final concentration of 0.04%.

Hydroxyapatite beads (HA) (BDH Biochemical, Ltd., Poole, England) were treated as previously described (Clark et al., 1978). Briefly, 10 mg of beads were washed and hydrated in distilled water in 250 microliter plastic microfuge tubes followed by equilibration overnight with adsorption buffer (0.05 M KCl, 1 mM K_2HPO_4, pH 7.3, 1 mM $CaCl_2$

and 0.1 mM $MgCl_2$). The beads were incubated with 200 microliters of saliva for 24 hours at 4°C and then washed with sterile adsorption buffer to remove nonadsorbing material. Control tubes without HA were treated identically.

B. gingivalis 381 cells were labeled by growth to late log phase in medium supplemented with (^3H) thymidine (10 mCi/ml). The cells were pelleted, washed twice in adsorption buffer, and dispersed with three 10-second pulses (medium power) with a microultrasonic cell disrupter.

The dispersed cells were mixed with each antiserum (1:100 dilution) and normal rabbit serum to a final concentration of 4×10^6 cell/ml. The cell-antiserum suspensions (200 microliters) were then added to the SHA beads in microfuge tubes and the tubes were rotated in an anaerobic chamber for 1 hour. Labeled cells alone (no antisera) were treated in the same manner to determine the number of cells adhering to the SHA surface. A control tube containing cells but no SHA was tested to quantitate the amount of cells bound to the tubes rather than to the SHA. One hundred microliters of adsorption buffer containing unadhered cells was removed and placed in minivials containing 3 ml of aqueous scintillation cocktail (Amersham/Searle, Arlington Heights, IL), and counted with a Scintillation Counter (Model 455 Parkard Tricarb). Determination of the number of cells adhering to the SHA was done by subtracting the number of cells (no. of counts) in solution from the total number of cells (no. of counts) which did not adhere to the tube.

Direct Hemagglutination Assay

The hemagglutination assays were carried out in V-bottom microtiter plates (Dynatech Laboratories, Inc., Alexandria, Virginia). Erythrocytes (sheep or human group O) were washed 3 times with PBS (0.02 M phosphate buffered saline), pH 7.2, and resuspended to a final concentration of 0.2% (v/v). Cells of *B. gingivalis* and antigen-expressing clones were washed twice in PBS and resuspended to an optical density of 0.5 and 2.0, respectively, at 660 nm. The cell suspensions were diluted in a twofold series with PBS and 0.05 ml of the suspensions were added to the wells. *E. coli* JM 109 (pUC 9) which was prepared in the same manner as the antigen-expressing clones, was included as a control. An equal volume (0.05 ml) of washed erythrocytes was added and mixed with the bacterial cells. The plates were stored for 16 hours at 4 °C and then examined for evidence of hemagglutination as follows. Agglutinated erythrocytes will settle as clumps which will be dispersed throughout the bottom of the wells, resulting in a pinkish-red coating of each well. In the absence of hemagglutination, the erythrocytes will settle on the bottom of the well as a central, smooth, bright red round disk. The titer was expressed as the reciprocal of the highest dilution showing positive agglutination.

Hemagglutination Inhibition Assay

The hemagglutination inhibition assay was also carried out in V-bottom microtiter plates. *B. gingivalis* cell suspensions in PBS were adjusted to the optical density of 0.5 at 660 nm. Each antiserum examined for hemagglutination inhibition activity was diluted two-fold

in a series of wells. Fifty µl of the bacterial suspension with twice the minimum number of cells which produced hemagglutination was then added to each well. After incubation with gentle shaking at room temperature for 1 hour, 0.05 ml of the washed erythrocytes were added to each well and mixed. The plates are left for 16 hours at 4°C and read for hemagglutination as described above for the hemagglutination assay. The titer was expressed as the reciprocal of the highest dilution showing hemagglutination inhibition.

Preparation of Antisera to Hemagglutinable *E. coli*

E. coli transformants which were able to agglutinate erythrocytes were grown in LB broth containing ampicillin as described above. Two rabbits were injected with each clone as described in Chapter Two. Sera were exhaustively adsorbed with *E. coli* JM 109 (pUC 9) and tested for anti-*B. gingivalis* activity by ELISA.

Adsorption of Anti-Clone 2 Antiserum

Anti-clone 2 antiserum diluted 1:10 was separately adsorbed with *B. gingivalis*, *E. coli* JM 109 (pUC 9), and clones 2, 5, and 7. Washed stationary phase cells of each bacterial culture were prepared as described in Chapter Two. For each adsorption, 10^7, 10^8, 10^9, and 10^{10} bacterial cells were mixed with 200 ul of serum and the suspensions were stored at 4°C overnight. The sera were recovered by centrifugation at 12,000 g for 10 minutes. Each adsorbed antiserum was assayed by ELISA to determine the titer to *B. gingivalis*.

DNA Procedures

Restriction endonuclease digestions of the recombinant plasmids from clones 2, 5, and 7 were performed according to manufacturer's directions. The size of DNA inserts were estimated and Southern blot analysis was performed as described in Chapter Two. Clone 5 DNA was digested with Hind III and two fragments of *B. gingivalis* inserts were isolated from agarose gels by the method of Zhu et al. (1985) employing centrifugal filtration of DNA fragments through a Millipore membrane inside a conical tip. The DNA preparations were extracted with phenol-chloroform, precipitated with ethanol and resuspended in TE, pH 8.0. Each DNA fragment was ligated to Hind III digested pUC 9 and the resulting recombinant plasmids were transformed into competent *E. coli* JM 109 cells as described in Chapter Two. Recombinant plasmids from these transformants were isolated by rapid plasmid DNA isolation (Silhavy et al., 1984), digested with appropriate restriction endonucleases, and analyzed by agarose gel electrophoresis.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis

B. gingivalis cell lysate and cells of *E. coli* transformant were prepared and analyzed by SDS-PAGE and Western blot techniques as described in Chapter Two. Antisera to clones 2, 5, and 7 exhaustively adsorbed with *E. coli* JM 109 (pUC 9) were used as probes in the Western blot. Control antisera included anti-clone 2 antiserum also adsorbed with *B. gingivalis* at the ratio of 10^{10} cells per 100 ul of antiserum, and antiserum to *E. coli* JM 109 harboring pUC 9 with *Eikenella corrodens* DNA insert.

Results

Assay for SHA Adhesin

It is possible that if the *B. gingivalis* SHA adhesin is expressed in *E. coli*, it may be expressed in a functionally inactive form, due to spacial interference by other *E. coli* surface structures, or it may not be processed adequately by the *E. coli* protein translocation machinery and thus may not be properly expressed on the *E. coli* surface. However, there is a strong possibility that the expressed antigen would still be antigenically intact. Thus, anti-*B. gingivalis* 381 antiserum which inhibits the adherence of *B. gingivalis* 381 to SHA was adsorbed with each antigen-expressing clone until the titer of this antiserum to each clone was reduced to zero. Each adsorbed antiserum was tested for inhibition of *B. gingivalis* adherence to SHA. If a clone expresses an antigenically active adhesin, the adsorbed antiserum should be unable to inhibit *B. gingivalis* 381 adherence to SHA or may partially inhibit the adherence.

The results in Table 3 summarize the SHA inhibition data and indicate that the antiserum adsorbed with each antigen-expressing clone still inhibited the adherence of *B. gingivalis*. There is no apparent significant decrease in the percent inhibition by each adsorbed antiserum.

Assay for Hemagglutinin

The rationale to identify the clones which express hemagglutinin were analogous to those described for the SHA adhesin. The anti-*B. gingivalis* antiserum adsorbed with each antigen-expressing clone and *E.*

Table 3. Inhibition of adherence to SHA by adsorbed anti-*B. gingivalis* antisera

Inhibitor and dilution		% adherence[a]	% inhibition[b]
None		83.35	-
Normal rabbit serum	1:100	80.08	0.05
Antiserum unadsorbed	1:100	22.70	72.15
Antiserum adsorbed with:			
E. coli JM 109 (pUC 9)	1:100	21.57	73.07
Clone 2	1:100	10.73	86.59
Clone 3	1:100	22.60	71.78
Clone 4	1:100	16.24	79.71
Clone 5	1:100	27.37	65.82
Clone 7	1:100	19.90	75.15

[a] Percent adherence was calculated from the following formula: % adherence = [(CPM from tube without SHA - CPM from tube with SHA)/(CPM from tube without SHA)] x 100.

[b] Percent inhibition was calculated from the following formula: % inhibition = [1 - (% adherence in the presence of antibody / % adherence in the absence of antibody)] x 100.

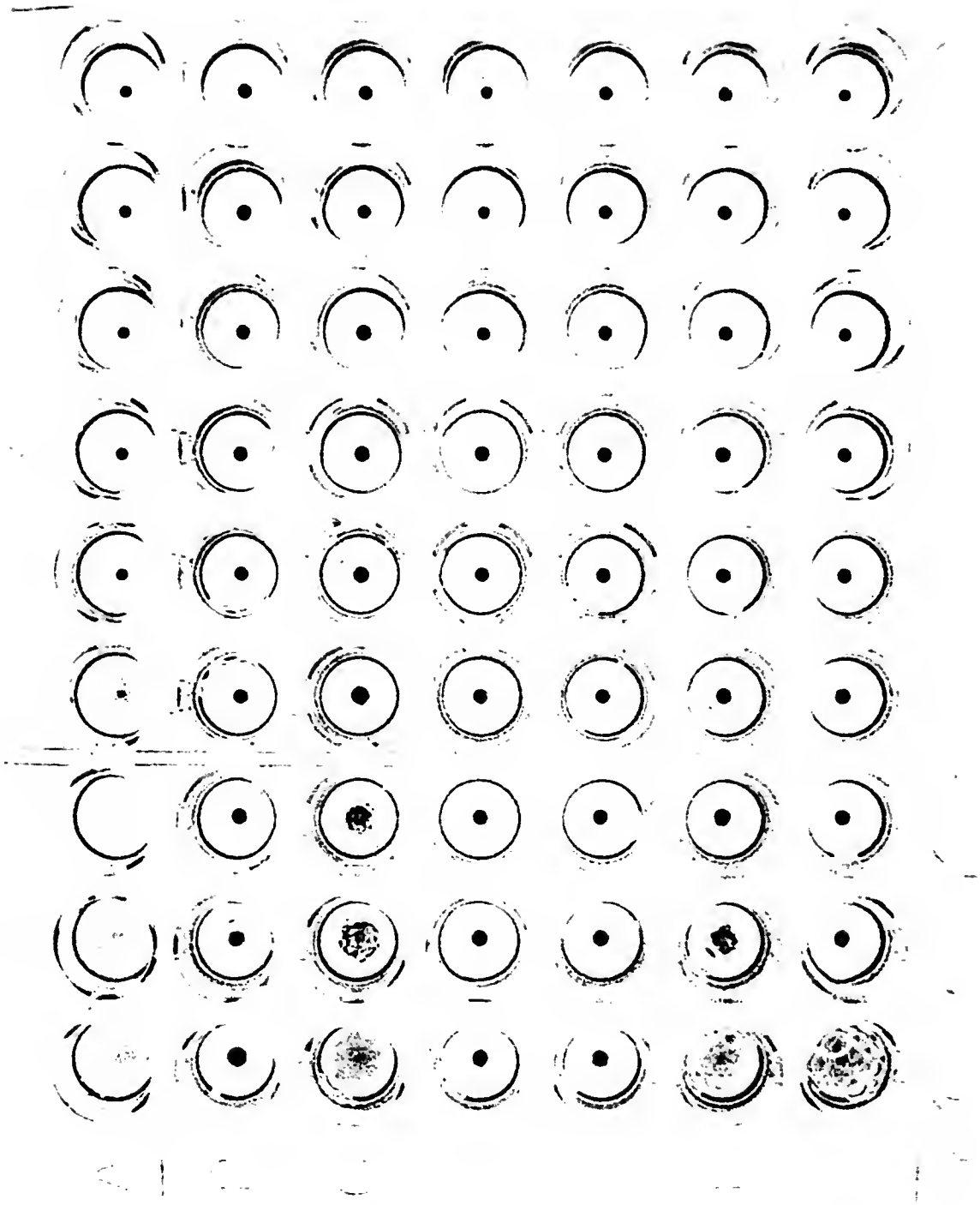
coli JM 109 (pUC 9), as described for the SHA assay, were tested for removal of hemagglutination inhibition activity of anti-*B. gingivalis* antiserum. Since it is necessary to determine the minimum number of *B. gingivalis* cells which produces hemagglutination before performing the hemagglutination inhibition assay, a direct hemagglutination assay of antigen-expressing clones together with *B. gingivalis* was first performed.

The direct hemagglutination assay of these clones demonstrated that clones 2, 5, and 7 did agglutinate sheep erythrocytes, whereas *E. coli* JM 109 (pUC 9) did not (Figure 9). The hemagglutination titer of clone 2 was 2 and that of clones 5 and 7 agglutinated erythrocytes at the undiluted suspension. In addition, clone 5 was found to auto-agglutinate when resuspended in PBS, pH 7.2.

Restriction Maps

Since three of the antigen-expressing clones were found to agglutinate erythrocytes, the possibility arose that they may have common DNA inserts which encode the same function. Clone 2 resulted from some kind of DNA rearrangement, i.e., clone 2 DNA when cut by Hind III, did not result in pUC 9 and insert bands but showed one large band and one small band as described in Chapter Two (Figure 2, lane 11). The rearrangement may have resulted from a deletion or other rearrangement at one Hind III end of the insert and another Hind III end may still be intact. In order to obtain information as to the nature of the rearrangement of clone 2 and the relationship of the three hemagglutinating clones to one another, restriction maps of these three clones were generated.

Figure 9. Hemagglutination of sheep erythrocytes. Bacterial suspensions were diluted in 2 fold serial dilutions and mixed with an equal volume of 0.2% (V/V) erythrocytes as described in the methods. Row 1 is the undiluted bacterial suspensions. (A) *B. gingivalis* 381, (B) *E. coli* JM 109 (pUC 9), (C) clone 2, (D) Clone 3, (E) Clone 4, (F) Clone 5, (G) Clone 7.



The recombinant plasmids of clones 2, 5, and 7 were restricted with several restriction endonucleases and analyzed in 1.2% agarose gels as shown in Figures 10, 11, and 12. A restriction map of each clone was generated as shown in Figures 13, 14, and 15. A schematic diagram of restriction enzyme recognition sites of these three clones is detailed in Figure 16. This data indicates that the clone 2 insert appears to be different from that of clones 5 and 7, whereas clones 5 and 7 have one insert fragment in common. The restriction map of clone 2 revealed that the Hind III site of the DNA insert at the amino terminal end of the β-galactosidase gene was still intact but a deletion occurred at the other end of the insert and included most of the linker. The linker region with recognition sites of Pst I, Sal I, Bam HI and Sma I was deleted but the Eco RI site was still intact as well as other sites upstream such as Pvu II and Nar I.

Southern Blot Analysis

To further confirm the results of the restriction maps, ^{32}P-labeled clone 7 recombinant DNA was used as a probe for hybridization of restricted recombinant plasmids by Southern blot analysis. Clone 2 DNA restricted with Hind III, Eco RI, and Sma I resulted in DNA fragments of pUC 9 and 4 pieces of insert of approximately 1,400, 1,300, 420, and 150 bp (Figure 17, panel A, lane 2). Clone 5 DNA restricted with Hind III resulted in fragments of pUC 9 and 2 pieces of insert of approximately 4,800 and 760 bp (Figure 17, panel A, lane 3). Fragment bands of pUC 9 and inserts of approximately 2,800, 2,000, and 760 bp were generated from digestion of clone 5 DNA with Hind III and Bam HI (Figure 17, panel A, lane 5). Clone 7 DNA

Figure 10. Agarose gel electrophoresis of restriction digests of the recombinant plasmid from clone 2.
Lanes: 1, DNA marker-Hind III digest of lambda DNA; 2, pUC 9 digested with Hind III; 3, pUC 9 digested with Pvu II; and recombinant plasmid from clone 2 digested with; 4, Hind III; 5, Pvu II; 6, Pvu II and Hind III; 7, Eco RI; 8, Eco RI and Hind III; 9, Sma I; 10, Sma I and Hind III; 11, Sma I and Eco RI; 12, Eco RI and Pvu II; 13, Nar I; 14, Eco RI and Nar I; 15, Bam HI; 16, Hind III and Bam HI; 17, Sma I and Bam HI; 18, Cla I; 19, Cla I and Hind III; 20, Eco RI and Cla I.

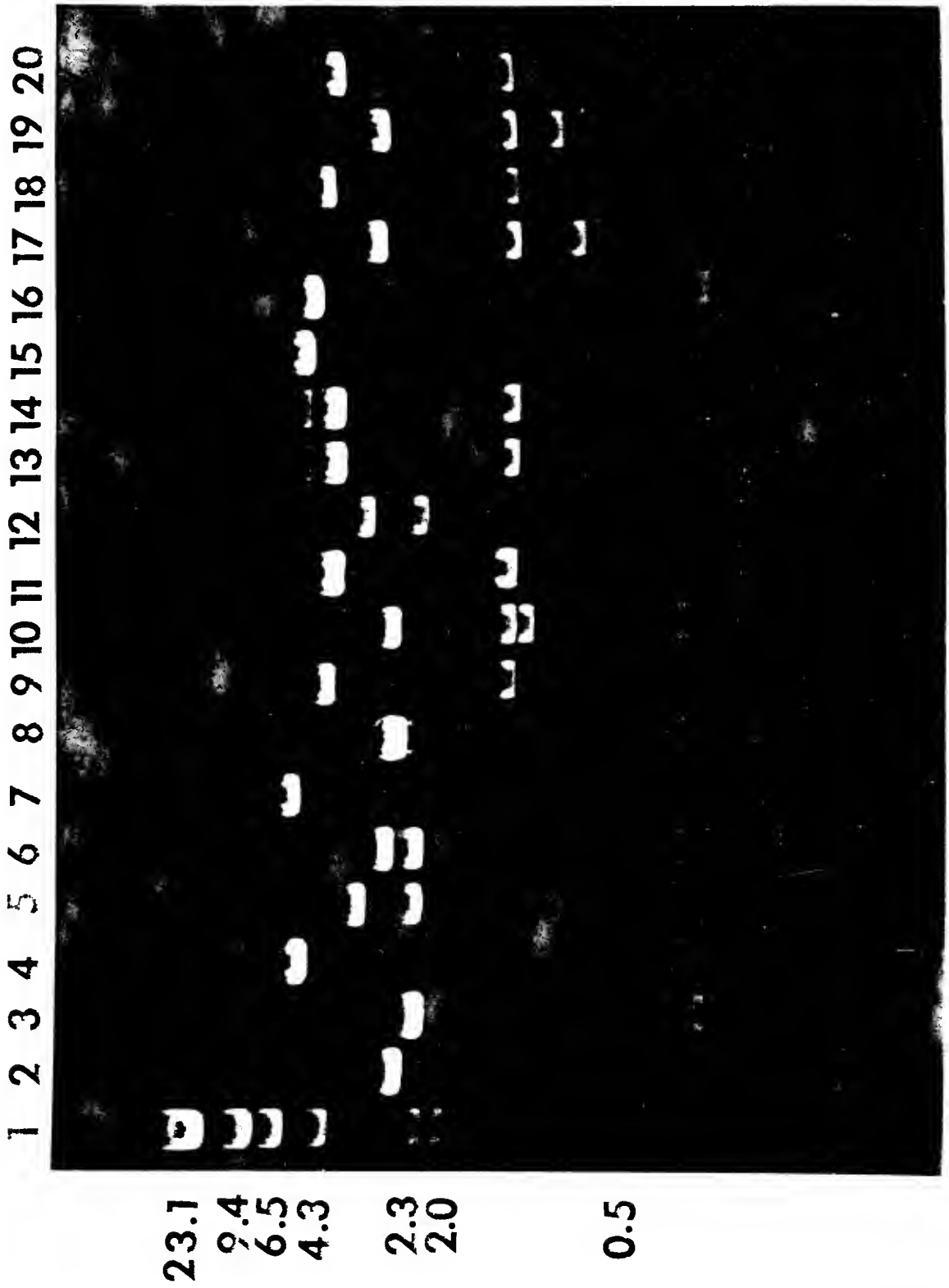

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
23.1
9.4
6.5
4.3
2.3
2.0
0.5

Figure 11. Agarose gel electrophoresis of restriction digests of the recombinant plasmid from clone 5.
Lanes: 1, DNA marker-Hind III digest of lambda DNA; 2, pUC 9 digested with Hind III; and recombinant plasmid from clone 5 digested with; 3, Hind III; 4, Bam HI; 5, Hind III and Bam HI; 6, Sal I; 7, Hind III and Sal I; 8, Hind III and Eco RV; 9, Stu I; 10, Hind III and Stu I; 11, Stu I and Sal I; 12, Stu I and Eco RV; 13, Stu I and Asp 718; 14, Hind III and Asp 718; 15, Bam HI and Asp 718; 16, Bam HI and Stu I; 17, Eco RI; 18, Hind III and Eco RI.

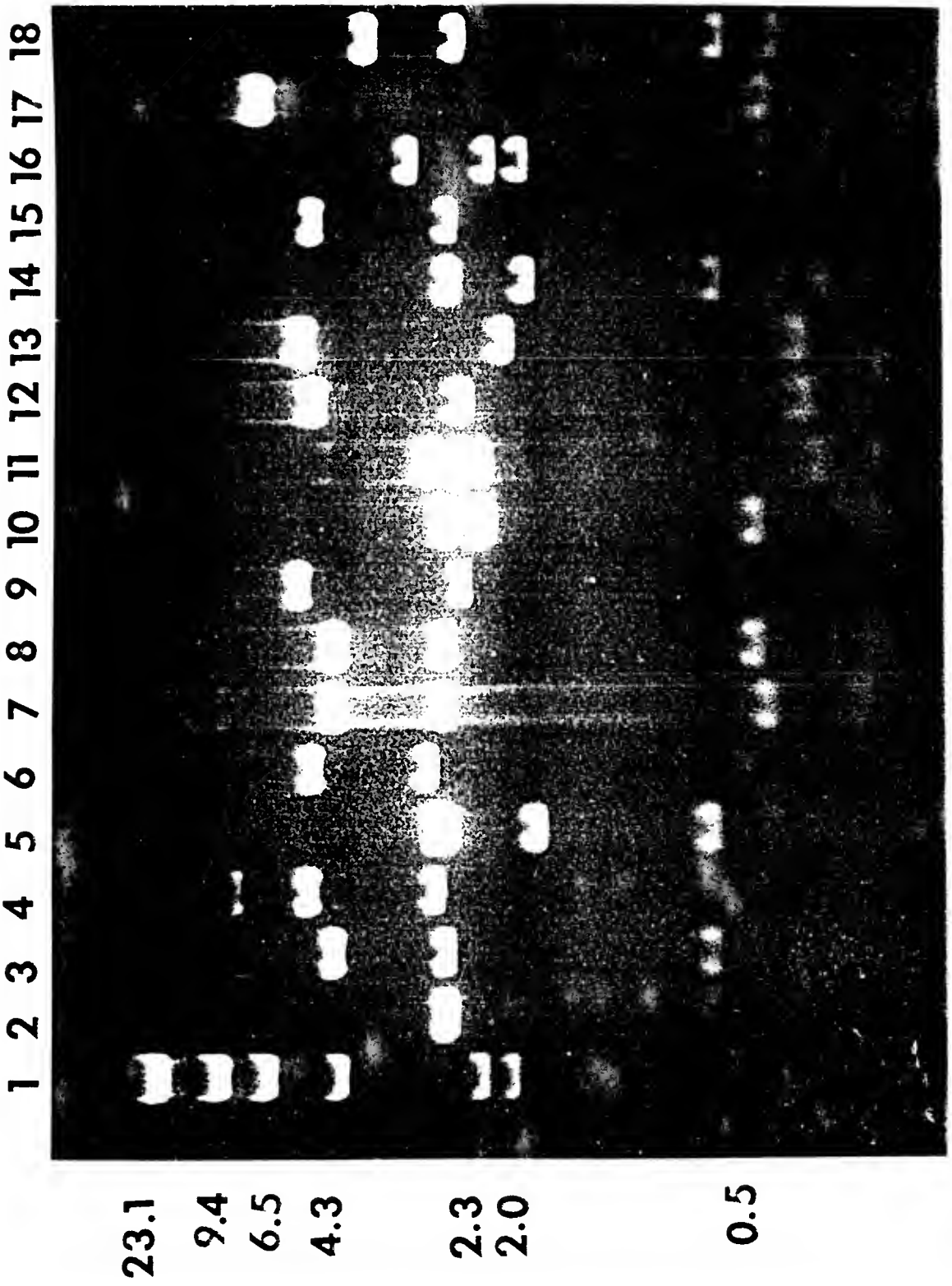

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
23.1
9.4
6.5
4.3
2.3
2.0
0.5

Figure 12. Agarose gel electrophoresis of restriction digests of recombinant plasmids from clones 5 and 7.
Lanes: 1, DNA marker-Hind III digest of lambda DNA; 2, pUC 9 digested with Hind III; recombinant plasmids from clones 5 and 7 in adjacent lanes digested with; 3 and 4, Hind III; 5 and 6, Hind III and Bam HI; 7 and 8, Hind III and Asp 718; 9 and 10, Hind III and Stu I; 11 and 12, Sal I; 13 and 14, Hind III and Eco RI.

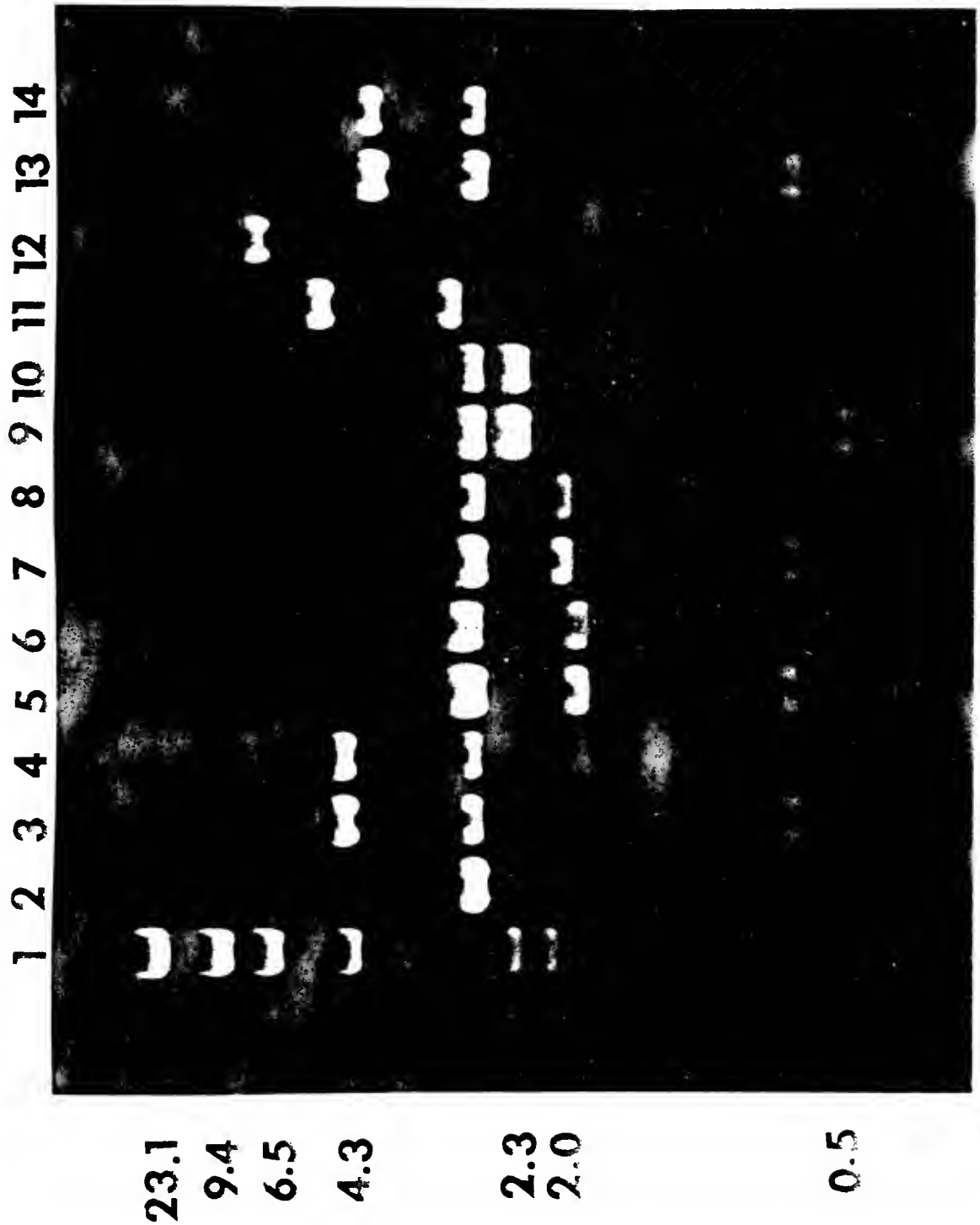

1 2 3 4 5 6 7 8 9 10 11 12 13 14
23.1
9.4
6.5
4.3
2.3
2.0
0.5

Figure 13. REstriction map of the recombinant plasmid from clone 2. The heavy line represents *B. gingivalis* DNA insert.

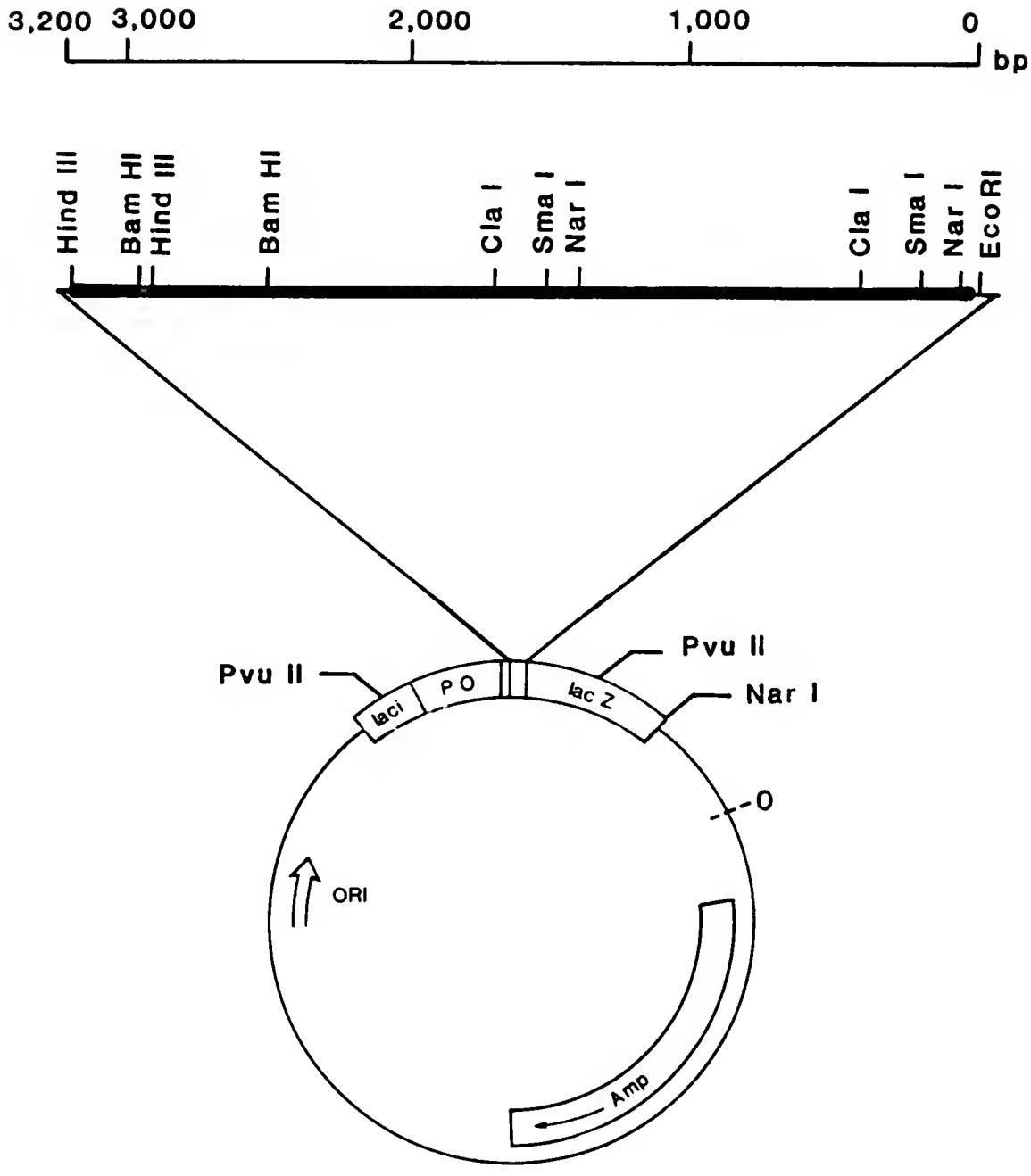

3,200
3,000
2,000
1,000
0
bp
Hind III
Bam HI
Hind III
Bam HI
Cla I
Sma I
Nar I
Cla I
Sma I
Nar I
EcoRI
Pvu II
Pvu II
Nar I
lacI
P O
lac Z
0
ORI
Amp

Figure 14. Restriction map of the recombinant plasmid from clone 5. The heavy line represents *B. gingivalis* DNA insert.

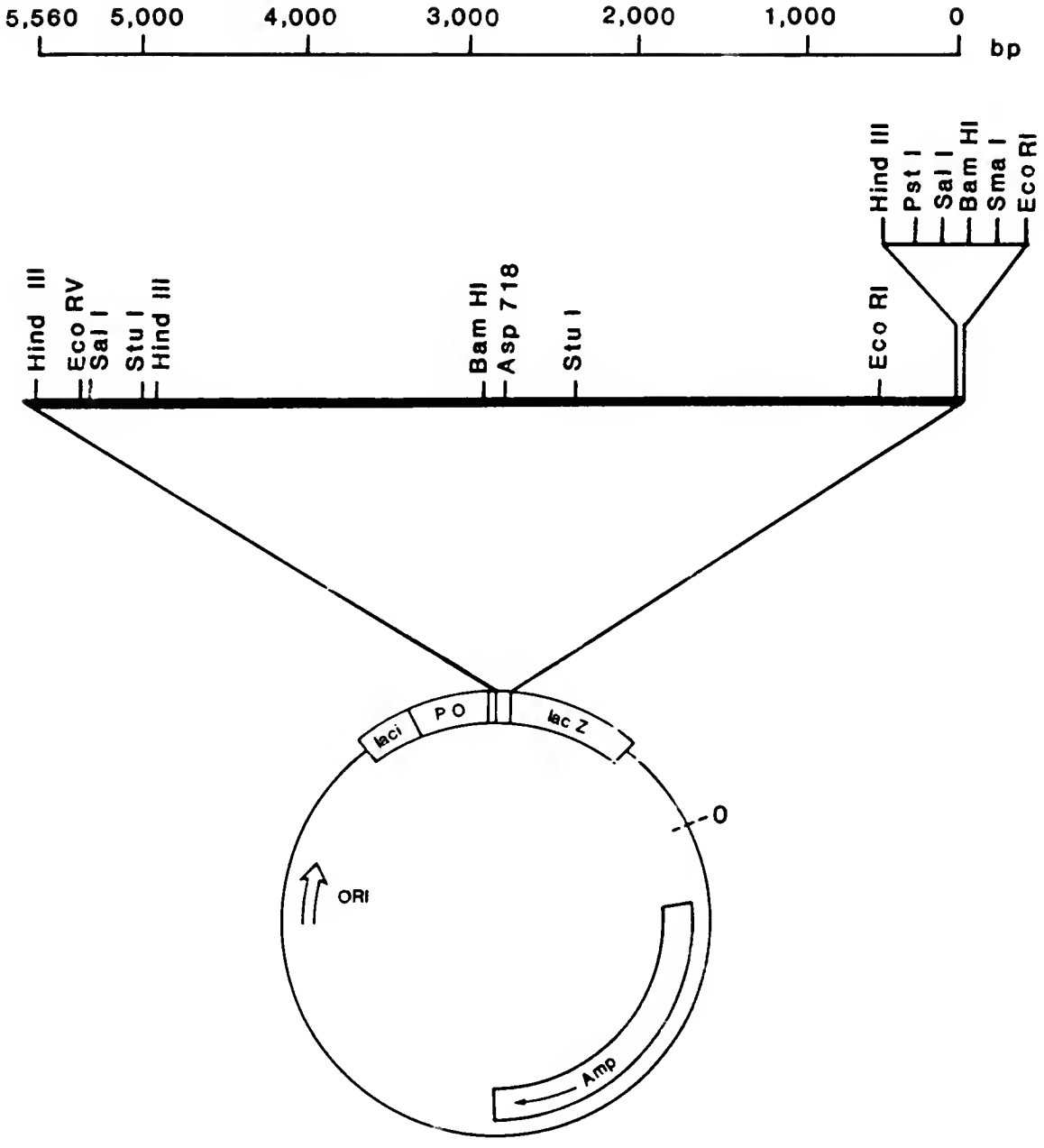

5,560
5,000
4,000
3,000
2,000
1,000
0
bp
Hind III
Pst I
Sal I
Bam HI
Sma I
Eco RI
Hind III
Eco RV
Sal I
Stu I
Hind III
Bam HI
Asp 718
Stu I
Eco RI
lacI
P O
lac Z
0
ORI
Amp

Figure 15. Restriction map of the recombinant plasmid from clone 7. The heavy line represents *B. gingivalis* DNA insert.

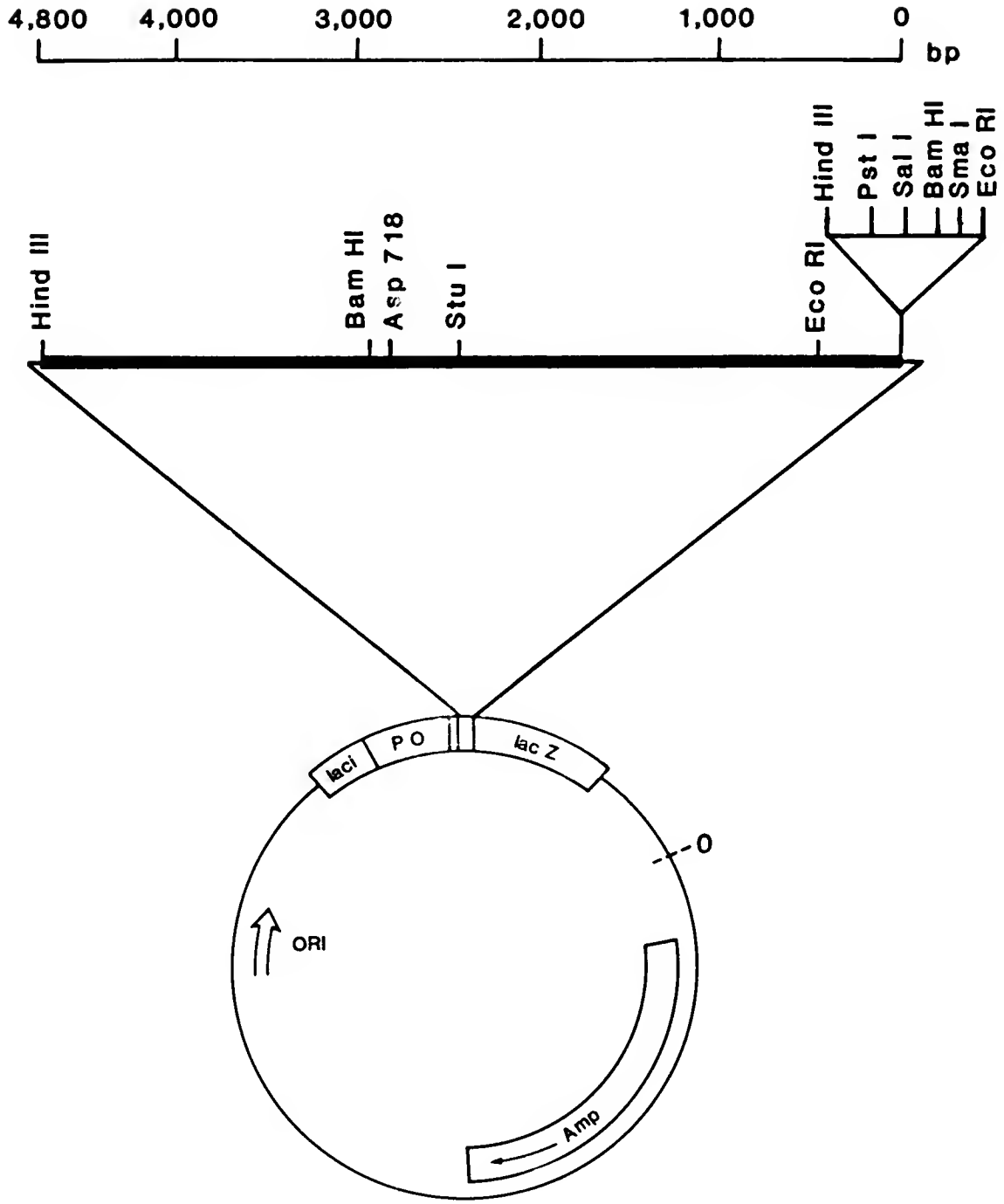

4,800
4,000
3,000
2,000
1,000
0
bp
Hind III
Bam HI
Asp 718
Stu I
Eco RI
Hind III
Pst I
Sal I
Bam HI
Sma I
Eco RI
lacI
P O
lac Z
0
ORI
Amp

Figure 16. Schematic diagram of restriction enzyme recognition sites of recombinant plasmids from clones 2, 5, and 7. The solid lines represent *B. gingivalis* DNA inserts. The hatched boxes represent pUC 9 regions.

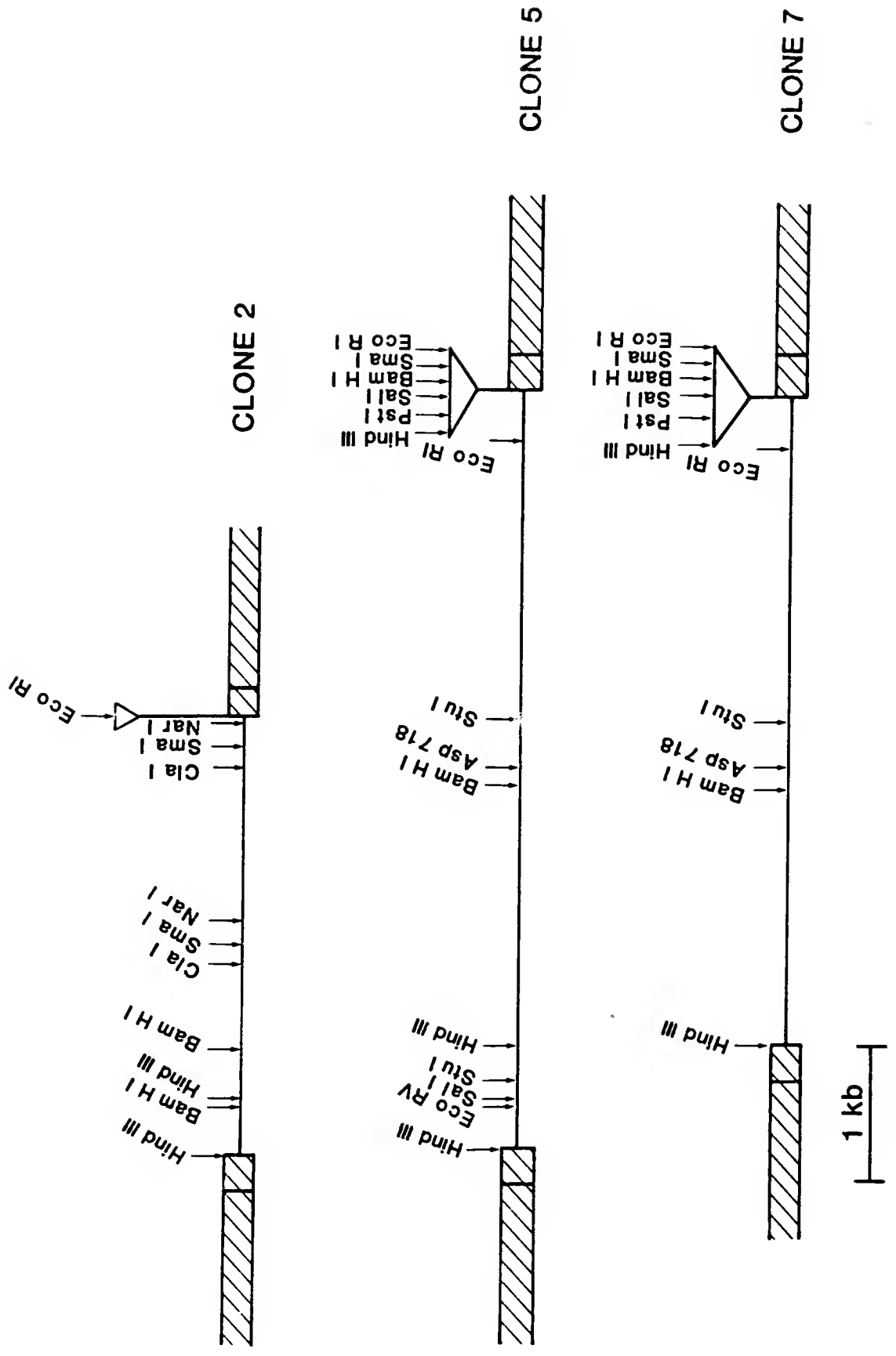

CLONE 2
Eco RI
Nar I
Sma I
Cla I
Nar I
Sma I
Cla I
Bam H I
Hind III
Bam H I
Hind III
CLONE 5
Eco RI
Hind III
Pst I
Sal I
Bam H I
Sma I
Eco R I
Stu I
Bam H I
Asp 718
Hind III
Eco RV
Sal I
Stu I
Hind III
CLONE 7
Eco RI
Hind III
Pst I
Sal I
Bam H I
Sma I
Eco R I
Stu I
Bam H I
Asp 718
Hind III
1 kb

Figure 17. Southern blot analysis of the hemagglutinating *E. coli*
(A) Agarose gel (1.2%) showing restriction digests of the DNA. Lanes: 1, pUC 9 digested with Hind III; 2, recombinant plasmid from clone 2 digested with Hind III, Eco RI, and Sma I; 3, recombinant plasmid from clone 5 digested with Hind III; 4, recombinant plasmid from clone 7 digested with Hind III; 5, recombinant plasmid from clone 5 digested with Hind III and Bam HI; 6, recombinant plasmid from clone 7 digested with Hind III and Bam HI.
(B) Autoradiograph of DNA in panel A after Southern transfer and hybridization with ^{32}P-labeled recombinant DNA from clone 7.

A

B

restricted with Hind III alone and Hind III together with Bam HI resulted in pUC 9 and an insert of 4,800 bp (Figure 17, panel A, lane 4), and pUC 9, insert of 2,800 and 2,000 bp (Figure 17, panel A, lane 6), respectively.

Hybridization of these transferred restricted DNAs demonstrated that the clone 7 probe hybridized to pUC 9 and the common insert of clones 5 and 7 but not to the insert of clone 2 (Figure 17, panel B).

Subcloning of Clone 5 for Autoagglutination and Hemagglutination

Clone 5 was found to agglutinate erythrocytes and autoagglutinate while clone 7 was only able to agglutinate erythrocytes. Clone 5 has an insert of 760 bp in addition to the common insert of 4,800 bp of clone 7. This data suggested that the 760 bp insert might encode for the autoagglutinating activity and the 4,800 bp fragment for the hemagglutinating activity of clone 5. The recombinant plasmid of clone 5 was thus digested with Hind III to generate pUC 9 and inserts of 4,800 and 760 bp. Each insert band was isolated from the agarose gel and ligated to Hind III cut pUC 9 and transformed into *E. coli* JM 109. The plasmids were isolated from these transformants and digested with restriction endonucleases. Subclones with different orientations of the insert were obtained. Subclones of 760 bp inserts were designated clone 5.1 and 5.2 and the subclones of 4,800 bp inserts, clone 5.3 and 5.4. Recombinant plasmids of clones 5.1 and 5.2 digested with Hind III did result in pUC 9 and the 760 bp inserts (Figure 18, lanes 2 and 3), and different patterns of restricted DNAs were seen when digested with Sal I (Figure 18, lanes 6 and 7). Hind III-restricted recombinant plasmids of clones 5.3 and 5.4 revealed pUC 9

and inserts of 4,800 bp (Figure 18, lanes 4 and 5), while Eco RI-restricted recombinant plasmids showed different patterns (Figure 18, lanes 8 and 9). Both clones 5.1 and 5.2 were able to autoagglutinate when resuspended in PBS, pH 7.2, but could not agglutinate erythrocytes. Clones 5.3 and 5.4 were both able to agglutinate erythrocytes but did not autoagglutinate.

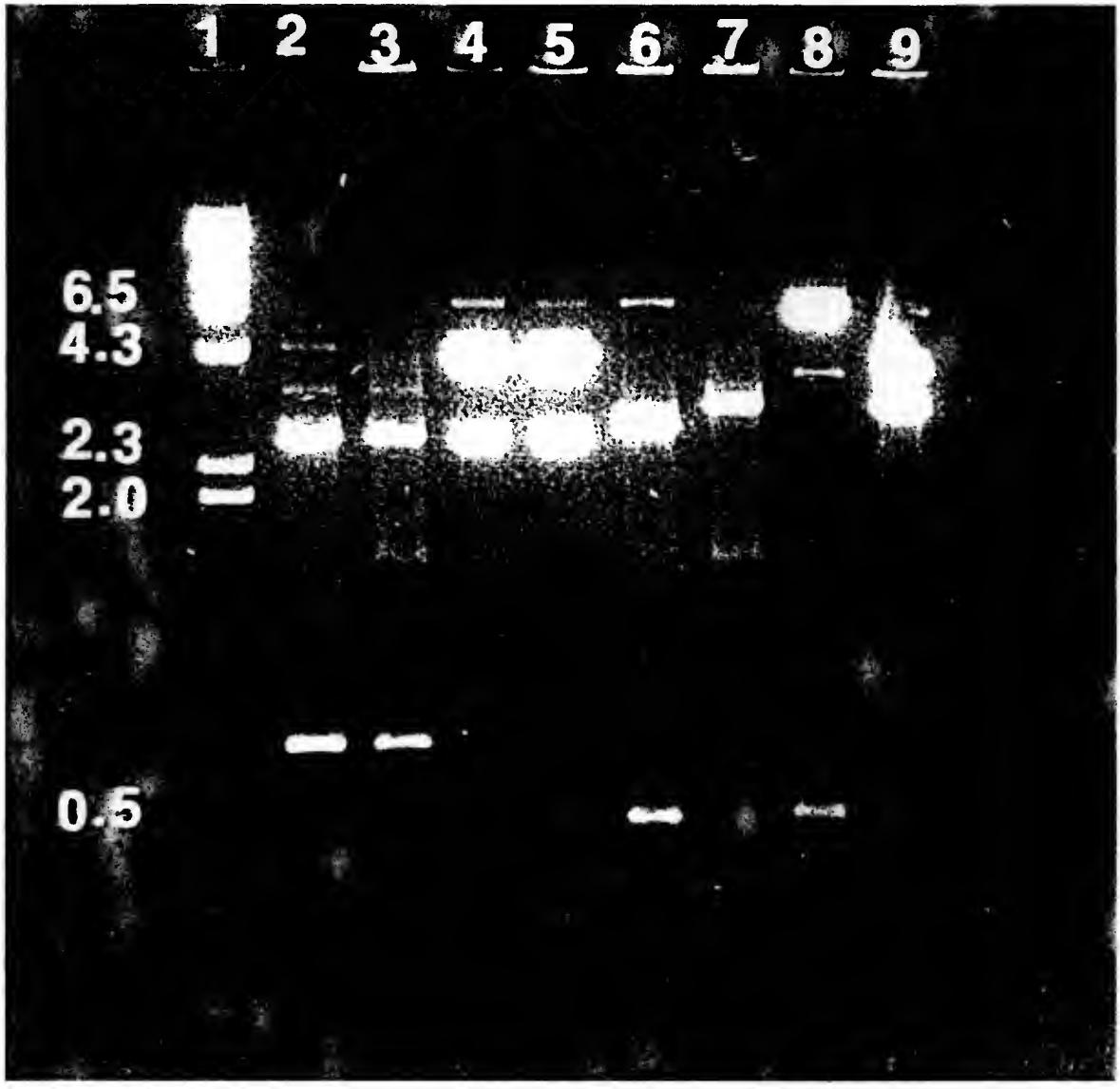
Western Blot Analysis of *B. gingivalis* Antigens Synthesized in hemagglutinable *E. coli*

Upon Western blot analysis of clone 2, a protein antigen of approximately 125 K and a smear of lower molecular weight were detected using *E. coli* adsorbed anti-*B. gingivalis* antiserum but antigens expressed in clones 5 and 7 were not detected by Western blot analysis (described in Chapter Two). In an attempt to detect antigen expression of DNA inserts in clones 5 and 7 and to achieve expression of a more stable product from clone 2, the recombinant plasmids from these clones were transformed into *E. coli* LC 137 [*htpR*(AmTs) *lonR 9* (Ts) *lac*(Am) *trp*(Am) *pho*(Am) *rpsL supC*(Ts) *mal*(Am) *tsx*::Tn10] kindly provided by A. L. Goldberg. This bacterial strain has mutations in the *htpR* and *lon* genes, the products of which are involved in intracellular protein degradation. However, this attempt was not successful since the expressed antigen of clone 2 was still degraded and antigen expression of clones 5 and 7 was not detected.

Identification of Native *B. gingivalis* Antigens

In order to determine the native *B. gingivalis* antigens which clone 2 expressed, antisera against clone 2 were made in rabbits for

Figure 18. Agarose gel electrophoresis of recombinant plasmids from clones 5.1, 5.2, 5.3, and 5.4.
Lanes: 1, DNA marker-Hind III digest of lambda DNA; 2 - 5, recombinant plasmids from clones 5.1, 5.2, 5.3, and 5.4 digested with Hind III; 6 and 7, recombinant plasmids from clones 5.1 and 5.2 digested with Sal I; 8 and 9, recombinant plasmids from clones 5.3 and 5.4 digested with Eco RI.


1 2 3 4 5 6 7 8 9
6.5
4.3
2.3
2.0
0.5

use as a probe in Western blot analysis. Pooled anti-clone 2 antiserum had a titer of 1:16,000 against *B. gingivalis* whole cell antigen. This antiserum was adsorbed exhaustively with *E. coli* JM 109 (pUC 9) until the anti-*E. coli* titer was reduced from 1:50,000 to 1:10 in the *E. coli* whole cell ELISA. The adsorbed antiserum, diluted to 1:200, was used as a probe to detect antigens separated in a 12.5% SDS polyacrylamide gel and transferred to a nitrocellulose sheet. As can be seen in Figure 19, this antiserum reacted with 2 major bands of approximately MWs 43,000 and 38,000 and 2 bands of MWs 32,000 and 30,000 in *B. gingivalis* cell lysate antigen and the 125 K protein band of expressed antigen in clone 2. Normal rabbit serum reacted to a common 40,000 molecular weight band of all the clones and *E. coli* JM 109 (pUC 9).

In order to prove that the *B. gingivalis* reactive polypeptides are exclusively *B. gingivalis* proteins, the native *B. gingivalis* antigens were reacted to *E. coli* adsorbed anti-clone 2 antiserum, *B. gingivalis* cell lysate antigen and clone 2 whole cell antigen were again separated in 12.5% SDS polyacrylamide gel. Upon transfer to a nitrocellulose sheet, each was reacted with 1) *E. coli* adsorbed anti-clone 2 antiserum, 2) *B. gingivalis* adsorbed anti-clone 2 antiserum, and 3) antisera to *E. coli* JM 109 harboring pUC 9 with an *Eikenella corrodens* DNA insert. As can be seen in Figure 20, *E. coli* adsorbed anti-clone 2 reacted to *B. gingivalis* cell lysate at 2 major bands of MWs 43,000 and 33,000, 2 bands of MWs 32,000 and 30,000 and 3 faint bands of higher molecular weight of approximately 110,000, 90,000, and 75,000 daltons. This adsorbed antiserum also reacted to a 125,000 MW band of expressed antigen in clone 2. *B. gingivalis* adsorbed anti-clone 2 and anti-*E. coli* JM 109 harboring pUC 9 with *Eikenella* DNA insert antisera did

Figure 19. Western blot analysis of native *B. gingivalis* antigens expressed by clone 2. Lanes: 1, *B. gingivalis* cell lysate (40 μg); 2 to 7, whole cell samples of clones 2, 3, 4, 5, 7, and *E. coli* JM 109 (pUC 9) as described in the methods.
(A) The blot was probed with *E. coli*-adsorbed anti-clone 2 antiserum.
(B) The blot was probed with normal rabbit serum.

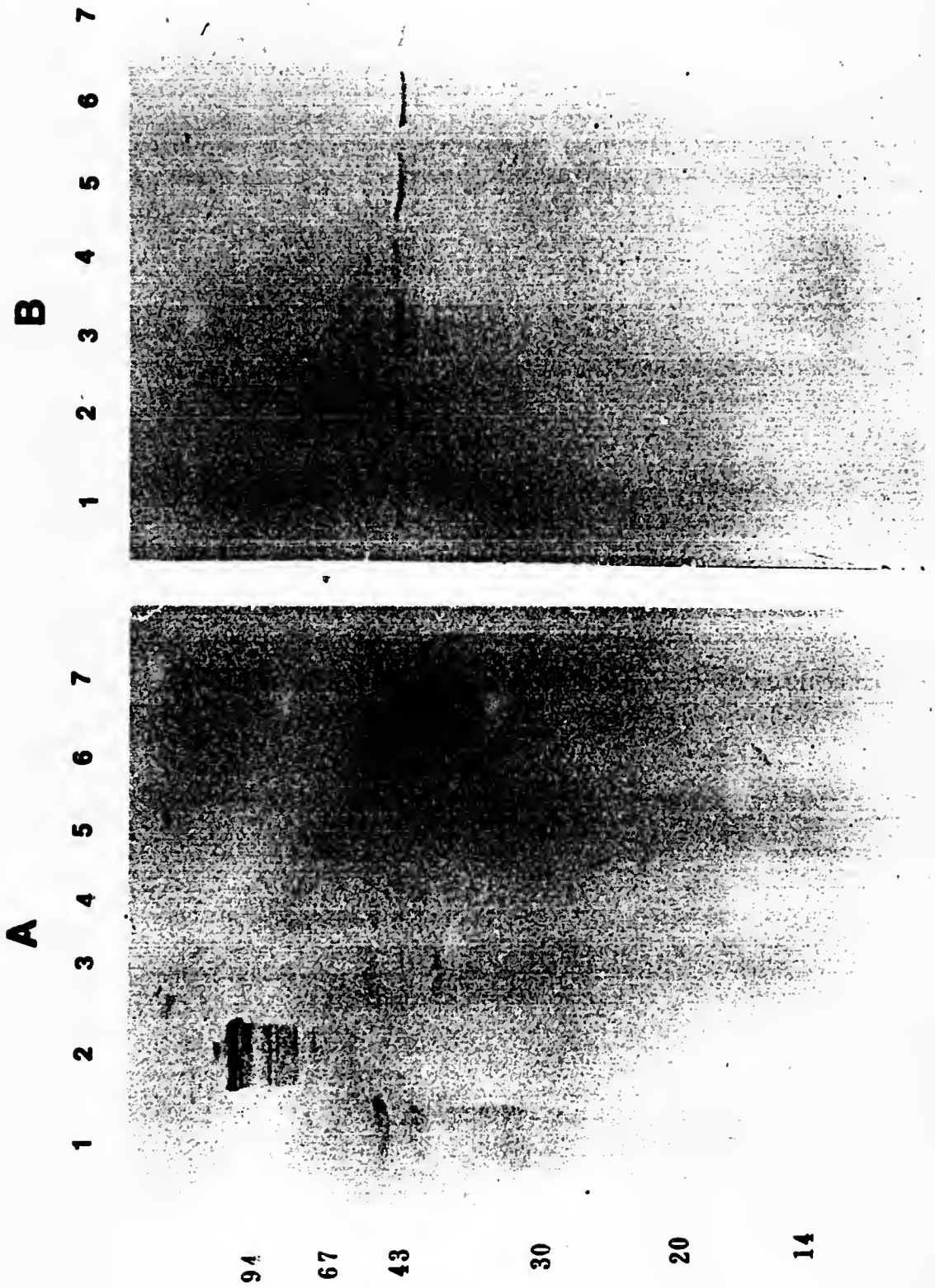
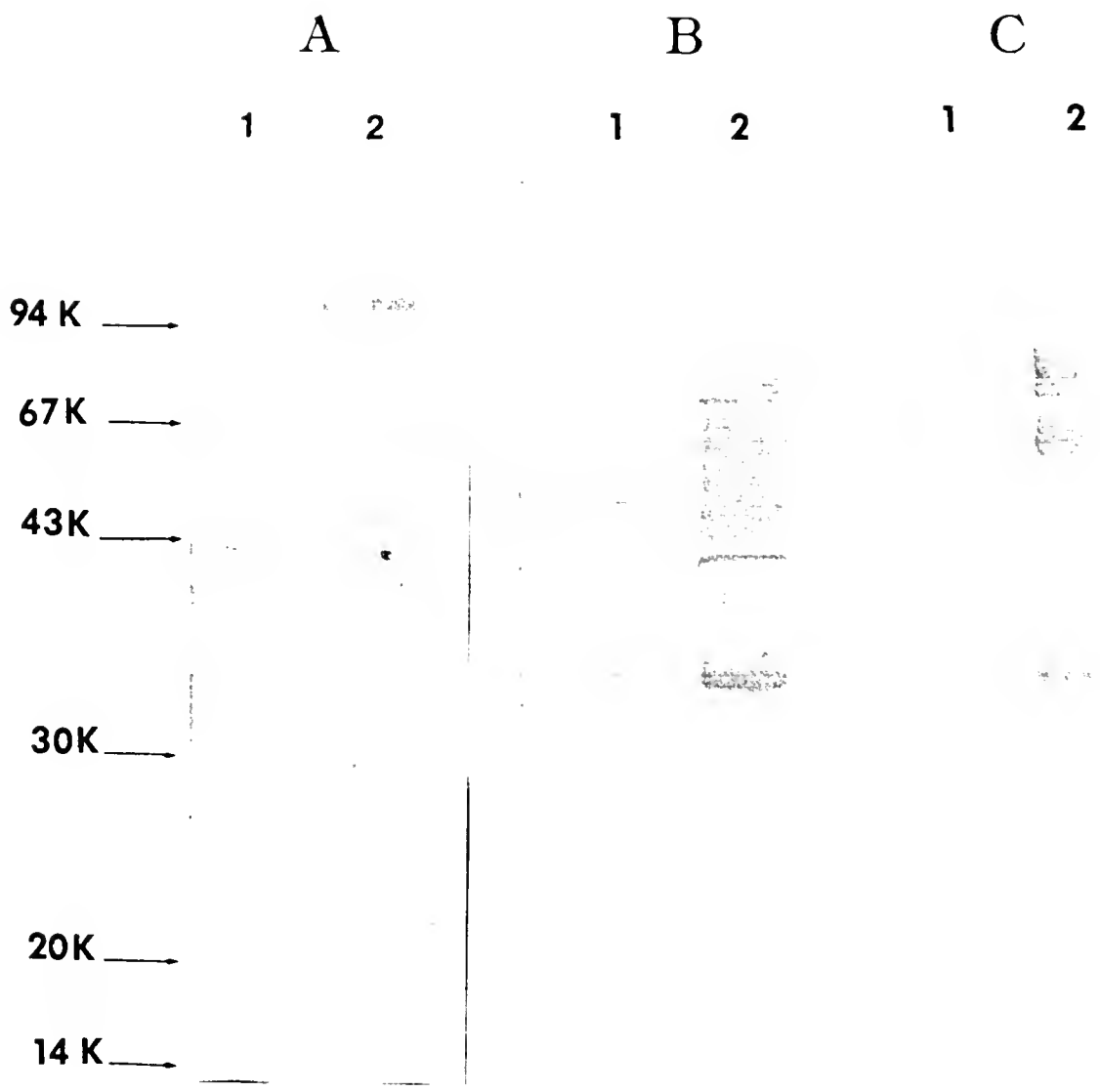

A
1 2 3 4 5 6 7
B
1 2 3 4 5 6 7
94
67
43
30
20
14

Figure 20. Western blot analysis of native *B. gingivalis* antigens expressed by clone 2.
Lanes: 1, *B. gingivalis* cell lysate (40 μg); 2, whole cell sample of clone 2.
(A) The blot was probed with *E. coli* adsorbed anti-clone 2 antiserum.
(B) The blot was probed with *B. gingivalis* adsorbed anti-clone 2 antiserum.
(C) The blot was probed with antiserum against *E. coli* JM 109 harboring pUC 9 with an *Eikenella* DNA insert.


A
B
C
1
2
1
2
1
2
94 K
67K
43K
30K
20K
14 K

not react to *B. gingivalis* antigens or to the expressed antigen of clone 2 but reacted with *E. coli* antigens in clone 2.

To define the native *B. gingivalis* antigens which clones 5 and 7 expressed, antisera against clones 5 and 7 were also made in rabbits and had titers of 1:800 and 1:1,600 to *B. gingivalis* antigens. These antisera exhaustively adsorbed with *E. coli* were used to identify the reactive native *B. gingivalis* antigens. Antisera against clones 5 and 7 at the dilution of 1:5 and 1:10 were found to react with 2 bands of approximately 43,000 and 38,000 daltons in *B. gingivalis* cell lysate antigen preparation but did not react to the expressed clone 2 antigen (Figure 21). This antiserum also reacted to a common band of approximately 36,000 daltons of *E. coli* antigen in each clone and *E. coli* JM 109 (pUC 9). Normal rabbit serum did not react to any *B. gingivalis* antigens (Figure 21).

In order to determine if the anti-clones 2, 5, and 7 antisera were reacting with the same *B. gingivalis* polypeptides or with different peptides of similar migration rates, four samples of *B. gingivalis* cell lysate antigens were separated in a 12.5% SDS polyacrylamide gel, transferred to nitrocellulose paper and reacted with anti-clone 2 antiserum diluted 1:200, anti-clone 5 antiserum diluted 1:5, anti-clone 7 antiserum diluted 1:10, and a mixture of anti-clones 2, 5, and 7 antisera at final concentrations of 1:200, 1:5, and 1:10, respectively. Any differences in the pattern of reaction were undiscernable in these 4 blots (Figure 22).

Figure 21. Western blot analysis of native *B. gingivalis* antigen expressed by clone 7. Lanes: 1, *B. gingivalis* cell lysate (40 μg); 2 to 5, whole cell samples of clones 2, 5, 7, and *E. coli* JM 109 (pUC 9) as described in the methods.
(A) The blot was probed with *E. coli*-adsorbed anti-clone 7 antiserum.
(B) The blot was probed with normal rabbit serum.

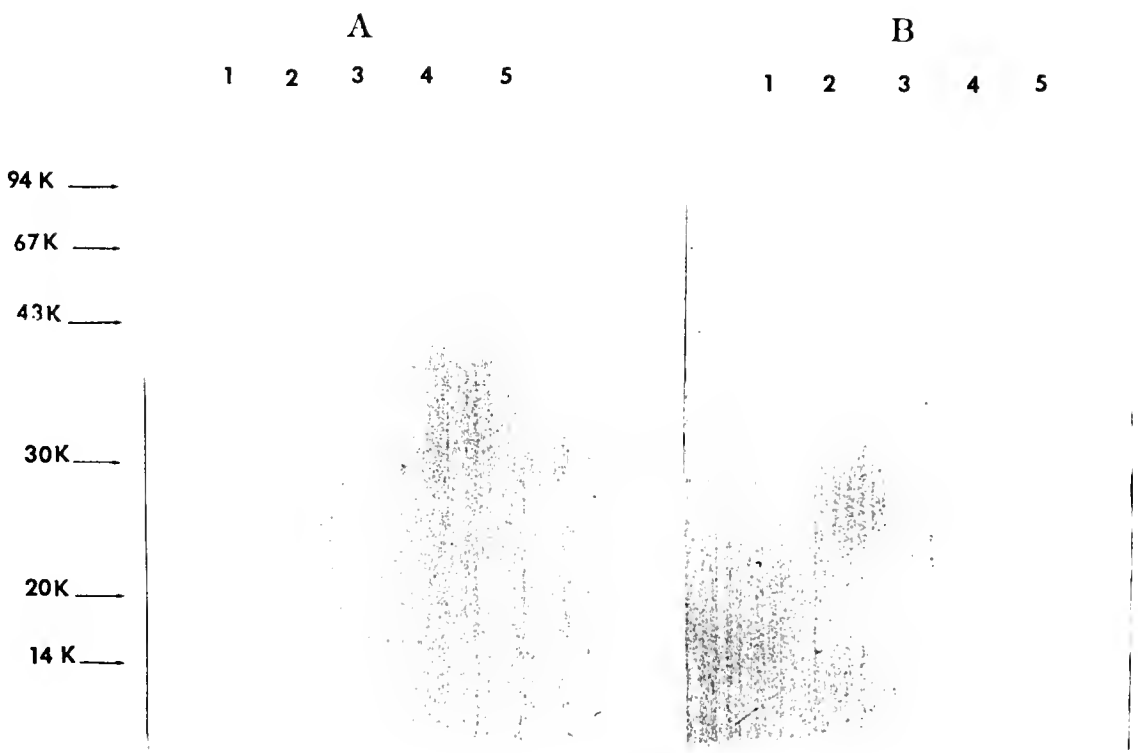
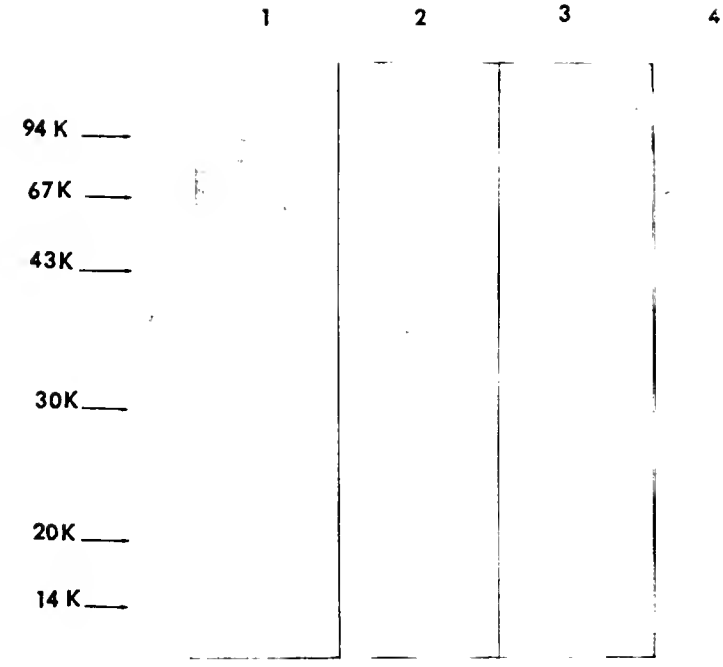

A
1 2 3 4 5
B
1 2 3 4 5
94 K
67K
43K
30K
20K
14 K

Figure 22. Western blot analysis of native *B. gingivalis* antigens expressed by clones 2, 5, and 7. Forty μg of *B. gingivalis* cell lysate was separated in a 12.5% SDS polyacrylamide gel, transferred to a nitrocellulose sheet, and probed with 1) *E. coli* adsorbed anti-clone 2 antiserum diluted 1:200; 2) *E. coli* adsorbed anti-clone 5 antiserum diluted 1:5, 3) *E. coli* adsorbed anti-clone 7 antiserum diluted 1:10; and 4) a mixture of the above antisera.


1
2
3
4
94 K
67K
43K
30K
20K
14 K

Determination of the Relationship between the Clones 2, 5, and 7 expressed antigens

Although antisera against clones 2, 5, and 7 reacted to *B. gingivalis* cell lysate at 2 major bands of 43,000 and 38,000 MWs (Figure 23, lane 1 of panel A, B, and C), *E. coli* adsorbed anti-clone 2 antiserum also reacted to the 125 K protein band synthesized in clone 2 (Figure 23, panel A, lane 2). However, *E. coli* adsorbed anti-clone 5 and anti-clone 7 antisera did not react to this expressed antigen band of clone 2 (Figure 23, lane 2 of panel B and C).

To further define the relationship of the epitopes of the expressed antigen in clone 2 from that of clones 5 and 7, adsorption of anti-clone 2 antiserum with several antigens was performed and each adsorbed anti-clone 2 antiserum was tested for its titer to *B. gingivalis* whole cell antigen by ELISA. These results were as shown in Figure 24. The antibody titer to *B. gingivalis* of anti-clone 2 antiserum was removed in a dose response manner by adsorption with *B. gingivalis* and clone 2 cells. Adsorption with *E. coli* JM 109 (pUC 9), clone 5 or clone 7 did not reduce the antibody titer to *B. gingivalis* of anti-clone 2 antiserum.

Hemagglutination Inhibition

The ability of antisera to *B. gingivalis* and hemagglutinable *E. coli* to inhibit the hemagglutinating activity of *B. gingivalis* was determined and is summarized in Table 4. All antisera inhibited *B. gingivalis* hemagglutination at titers 4 to 8 times that of normal rabbit sera.

Figure 23. Detection of *B. gingivalis* antigens synthesized by clones 2, 5, and 7 as determined by Western blot analysis. Lanes: 1, *B. gingivalis* cell lysate (40 μg); 2, whole cell sample of clone 2.
(A) The blot was probed with *E. coli* adsorbed anti-clone 2 diluted 1:200.
(B) The blot was probed with *E. coli* adsorbed anti-clone 5 diluted 1:5.
(C) The blot was probed with *E. coli* adsorbed anti-clone 7 diluted 1:10.

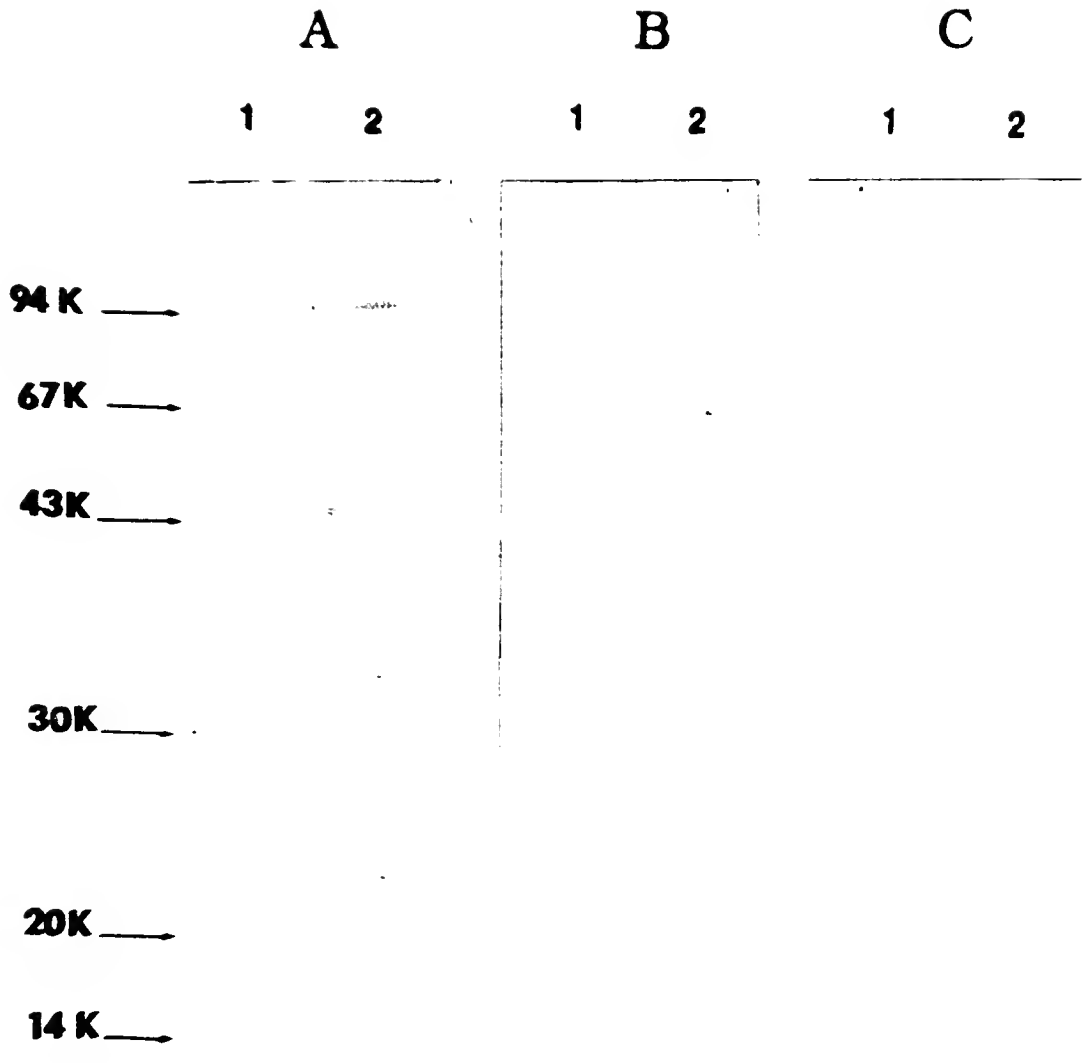

A
B
C
1
2
1
2
1
2
94 K
67K
43K
30K
20K
14 K

Figure 24. ELISA of anti-clone 2 antiserum adsorbed with various numbers of cells of *B. gingivalis* (○), *E. coli* JM 109 harboring pUC 9 (●), clone 2 (▪), clone 5 (△), and clone 7 (▲). The adsorbed antisera were added to *B. gingivalis*-coated microtiter plates and the assay was performed as described in the methods.

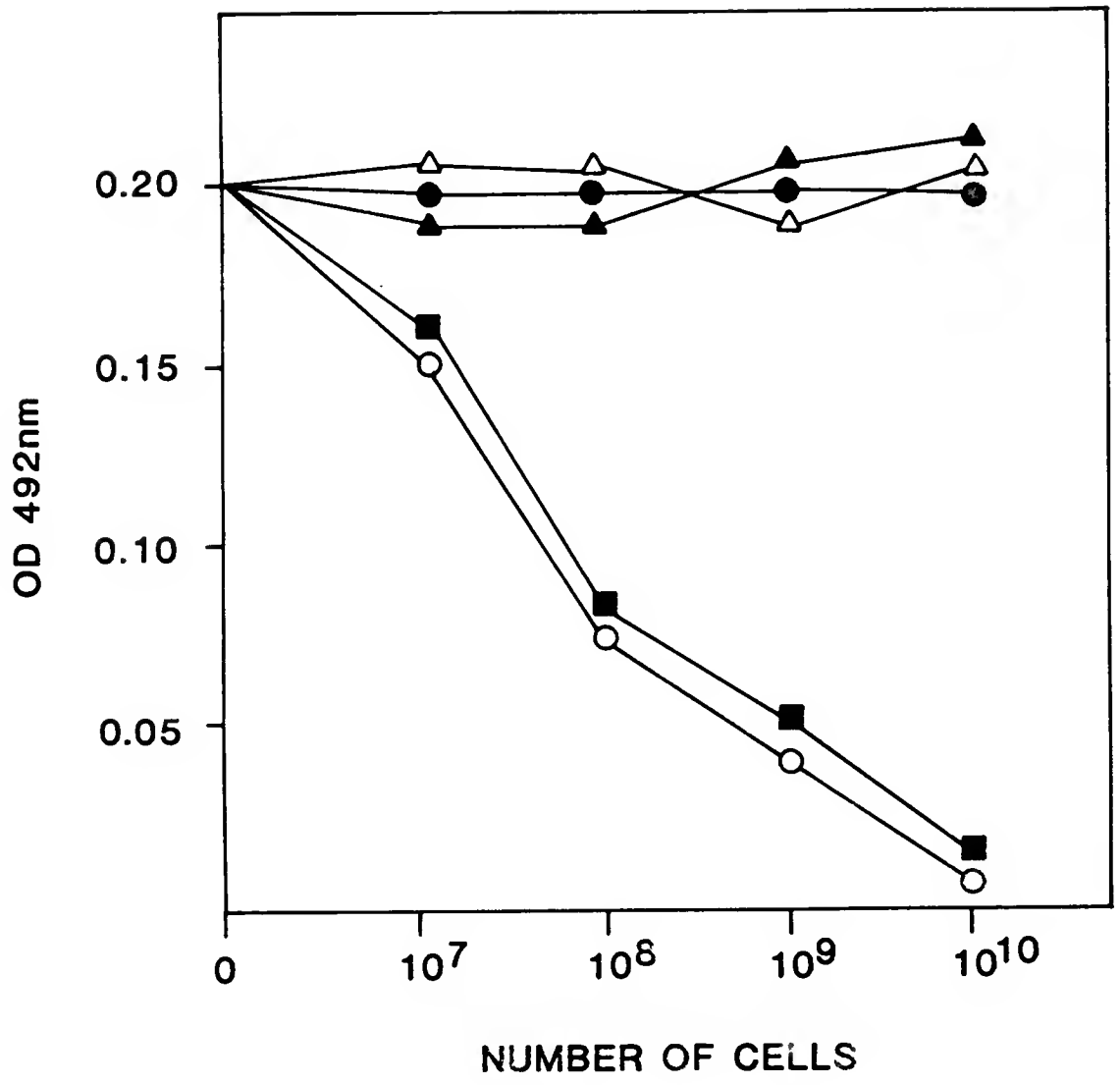

0.20
0.15
0.10
0.05
OD 492nm
0
10^7
10^8
10^9
10^10
NUMBER OF CELLS

Table 4. Inhibition of hemagglutinating activity of *B. gingivalis* by anti-hemagglutinating *E. coli* antisera.

Antiserum	Hemagglutination inhibition titer
Anti-*B. gingivalis*	
unadsorbed	640
adsorbed with *E. coli* JM 109 (pUC 9)	640
Normal rabbit serum[a]	160
Anti-clone 2	320 - 640
Preimmune	80
Anti-clone 5	160
Preimmune	40
Anti-clone 7	160
Preimmune	40

[a] Normal rabbit serum and preimmune sera titers are from each particular group of rabbits.

The ability of each hemagglutinating clone to remove the hemagglutination inhibition activity of anti-*B. gingivalis* antiserum was tested and the results are shown in Table 5. Each clone partially removed hemagglutination inhibition activity of anti-*B. gingivalis* antiserum. Clones 2 and 7 decreased the hemagglutination inhibition titer of *B. gingivalis* antiserum 2 to 4 fold. Adsorption of the antisera with clone 5 cells reduced the titer 4 to 8 fold and a mixture of these clones reduced the titer 8 fold. *B. gingivalis* itself reduced the titer 16 fold and *E. coli* JM 109 (pUC 9) had no effect on the inhibition activity of the antiserum.

Discussion

When antigen-expressing clones were surveyed for functional activities, clones 2, 5, and 7 were able to agglutinate erythrocytes whereas *E. coli* JM 109 (pUC 9) was not. The restriction maps and Southern blot hybridization of these clones indicated that clone 2 cells contain a different *Bacteroides* DNA insert from clones 5 and 7. Clone 5, which is also able to autoagglutinate, has a 760 bp DNA fragment in addition to a 4,800 bp fragment in common with the clone 7 insert. Subcloning of these 2 fragments in different orientations revealed that the 4,800 bp DNA encoded for the hemagglutinating activity and the 760 bp DNA for the autoagglutinating activity. Both fragments must contain a *Bacteroides* promoter since the subclones with opposite orientations of the inserts still express functional proteins. These data agree with the previous results described in Chapter Two indicating that antigen expression of clones 5 and 7 is not stimulated by IPTG. Unfortunately, *B. gingivalis* antigens expressed in clones 5

Table 5. Inhibition of hemagglutinating activity of *B. gingivalis* by adsorbed anti-*B. gingivalis* antiserum

Antiserum	Hemagglutination inhibition titer
Anti-*B. gingivalis*	
unadsorbed	640
adsorbed with *E. coli* JM 109 (pUC 9)	640
adsorbed with *B. gingivalis*	40
adsorbed with clone 2	160 - 320
adsorbed with clone 5	80 - 160
adsorbed with clone 7	160 - 320
adsorbed with clones 2, 5, and 7	80

and 7 could not be detected by Western blot analysis. Other techniques such as in vitro transcription-translation and immunoprecipitation might be appropriate to detect the expressed antigens.

To date, the hemagglutinin of *B. gingivalis* strain 381 has been partially purified in 2 different laboratories. The partially purified hemagglutinin isolated by Inoshita et al (1986) separated by SDS-PAGE demonstrated major protein bands of MWs 44 K, 37 K, and 24 K as well as bands at 30 K and greater than 100 K. Okuda et al. (1986) also purified a hemagglutinin, of which the SDS-PAGE pattern contained a major protein band of 40 K in addition to several protein bands in lesser amounts. A previous report by Naito et al. (1985) demonstrated that monoclonal antibody directed against *B. gingivalis* hemagglutinin (but not LPS or capsule), reacted with 40 K and 60 K bands of a hemagglutinin preparation. However, these bands appear to be approximately 40 K and 45 K, compared to the molecular weight standards in their blot. The Fab fragments of this monoclonal antibody were shown to inhibit the hemagglutinating activity of *B. gingivalis*. It thus appears that the *B. gingivalis* hemagglutinin may contain at least 2 protein bands as detected by SDS-PAGE. However, the structure and composition of the native molecule of *B. gingivalis* hemagglutinin is still unknown.

In the studies described here, *E. coli* adsorbed rabbit-polyclonal antibody against clone 2 was found to react with several bands in the *B. gingivalis* cell lysate preparation separated by SDS-PAGE. The most rapidly developing and strongest reaction appeared at 2 bands of 43 K and 38 K. Two bands of 32 K and 30 K appeared later and 3 faint bands of 110 K, 90 K, and 75 K sometimes were visible still later. This

strongly suggests that the *B. gingivalis* hemagglutinin is expressed in clone 2. The DNA insert of clone 2 might encode for all four of the polypeptides of 44 K, 38 K, 32 K, and 30 K or only a portion of some of these polypeptides of *B. gingivalis*. *E. coli* JM 109 has a nonsense suppressor tRNA, in which tRNA is able to suppress the termination codon (UAG) at the end of the gene that uses this codon. Therefore, the mRNA may be read through, resulting in the synthesis of the intact protein band of 125 K which might also contain a 10 K peptide of part of the β-galactosidase peptide. The 38 K polypeptide might be part of the 44 K peptide since it has been shown that monoclonal antibody reacts to 2 bands of similar molecular weights (Naito et al., 1985). The two peptides could be generated from a single gene by starting or terminating expression at different points. It is also possible that the clone 2 insert encodes the long polypeptides of approximately 105 K and 100 K and 90 K in *B. gingivalis*. These long peptides might be generated from a single gene by starting or stopping expression at different points. Once the cells of *B. gingivalis* are disrupted as prepared for cell lysate sample, the long polypeptides may be degraded into smaller peptides of 43 K, 38 K, 32 K, and 30 K. Therefore, the high molecular weight band in the *B. gingivalis* cell lysate to which antiserum against clone 2 reacted might be the precursor of the smaller polypeptides.

E. coli adsorbed rabbit-polyclonal antibody against clones 5 and 7 also reacted with 2 bands of 43 K and 38 K, but barely reacted with the higher bands of 110 K, 90 K, and 75 K and did not react with the bands of 32 K and 30 K. Thus, these clones and clone 2 contain nonhomologous DNA inserts and express different antigenic epitopes but all function

as hemagglutinins. Two possibilities that may explain this phenomenon are (1) different *Bacteroides* components migrated in SDS-PAGE the same distance. This however, appears unlikely. To resolve these bands, a technique of two-dimensional gel electrophoresis must be performed. (2) each cloned insert encodes a different portion of the same polypeptide of *B. gingivalis* and each portion with different antigenic epitopes can function as a hemagglutination.

In the latter case, the clone 7 insert contains a *Bacteroides* promoter but the clone 2 insert does not. The 43 K and 38 K polypeptides might be generated from a single gene by starting (or terminating) expression at different points. The clone 7 insert or part of the insert would code for the N-terminus of the 43 K polypeptide and end some where at the N-terminus of the 38 K polypeptide. The clone 2 insert may code for the same C-terminus of the 43 K and 38 K polypeptides and might contain the structural gene for other peptide (32 K and 30 K) to which antiserum against clone 2 reacts.

Hemagglutination inhibition by antiserum against each clone might result from spatial interference, i.e., Fab portions of antibody molecules might bind to other antigens of *B. gingivalis* which are close to, but are not actually hemagglutinin antigens. Therefore, they can hinder hemagglutinins from binding erythrocytes. In order to exclude this possibility, Fab fragments should be prepared from the IgG of these antisera and tested for their ability to inhibit hemagglutination of *B. gingivalis*. However, the ability of each clone to partially remove the hemagglutination inhibition activity of anti-*B. gingivalis* antiserum indicates that there are more than one antigenic determinant which functions as a hemagglutinin. It may be either the case of 2

functional epitopes of the same polypeptide or the case of 2 different hemagglutinin components.

CHAPTER FOUR
CONCLUSION

The hemagglutinating activity of *B. gingivalis* has been studied as a parameter that affects the adherence of this organism in the periodontal pocket since *B. gingivalis* possesses fimbriae (Okuda and Takazoe, 1974; Slots and Gibbons, 1978) and many fimbriae of other bacterial species have hemagglutinating activity as well as the ability to adhere to a variety of host cells (Pearce and Buchanan, 1980). It has been recently reported that sera from patients with adult periodontitis possess high antibody levels to the *B. gingivalis* hemagglutinin. It is thus suggested that the adhesive surface structures such as hemagglutinin participate in *B. gingivalis* colonization and antigenic stimulation of the host (Naito et al., 1987). Hemagglutinating activity has also been shown to be a unique characteristic of oral strains of *B. asaccharolyticus* (presently *B. gingivalis*). Nonoral strains of *B. asaccharolyticus* do not have hemagglutinating activity (Slots and Genco, 1979). Components of bacteria which mediate attachment to host tissues include surface structures such as fimbriae, capsular material, lipopolysaccharide, and membrane-associated extracellular vesicles (Slots and Genco, 1984). Fimbrial preparations from *B. gingivalis* 381 have been found to possess strong hemagglutinating activity but LPS or polysaccharide apparently do not (Okuda et al., 1981). However, Boyd and McBride (1984) have

reported that an outer membrane preparation of *B. gingivalis* W12 mediates hemagglutination but does not contain fimbriae-like structures. In addition, Yoshimura et al. (1984) purified a novel type of fimbriae from *B. gingivalis* 381 which did not show hemagglutinating activity. Recently, Okuda et al. (1986) have demonstrated that the monoclonal antibody against *B. gingivalis*, which reacted to partially purified hemagglutinin but not to LPS or capsule, bound to fibrillar structures of *B. gingivalis*. It is thus unclear whether the *B. gingivalis* hemagglutinin is present on fimbriae or not.

The hemagglutinin of *B. gingivalis* has not yet been completely purified and characterized. However, a few investigations have reported the isolation of hemagglutinin activity from *B. gingivalis*. Boyd and McBride (1984) prepared an outer membrane component containing hemagglutinating activity from *B. gingivalis* W12. This preparation contained three major proteins with molecular weights of 69,000, 41,500, and 22,000. Inoshita et al. (1986) isolated hemagglutinating activity from culture supernatants of *B. gingivalis* 381. The isolated hemagglutinin component contains three major proteins with molecular weights of 24,000, 37,000, and 44,000. Okuda et al. (1986) also purified a hemagglutinin of *B. gingivalis* 381 from culture supernatant which appears to have vesicle or tubelike structures and is comprised mainly of a 40,000 molecular-weight protein. Their recent report indicated that sera from most patients with adult periodontitis reacts to the hemagglutination antigen at 43,000 and 57,000 molecular weights (Naito et al., 1987).

This report describes the cloning of *B. gingivalis* chromosomal DNA in *E. coli* which then enables the *E. coli* host to bind erythrocytes,

the reaction of antisera against hemagglutinable *E. coli* with *B. gingivalis* antigens which have been previously reported to be the major antigens in partially purified hemagglutinin, and the ability of each hemagglutinable *E. coli* to partially remove the hemagglutination activity of anti-*B. gingivalis* antiserum. These data strongly suggest that I have cloned *B. gingivalis* hemagglutinin genes into *E. coli*. In addition, the results indicate that two different *B. gingivalis* DNA sequences express different antigenic epitopes, both of which function as hemagglutinins. To determine whether these cloned DNA fragments are adjacent in *Bacteroides* chromosomal DNA (i.e., contained in one gene) or not, the following experiments should be performed.

1. Hybridization of restricted *B. gingivalis* chromosomal DNA with DNA inserts from clones 2 and 7. Each DNA insert should hybridize to the same chromosomal DNA fragment if these inserts are contiguous on the chromosomal DNA. Appropriate restriction enzymes should be used to digest chromosomal DNA completely in order to generate fragments which are not cut at a site between these inserts. For partial digestion, conditions of digestion of the DNA must be adjusted in order to maximize the possibility of obtaining similar hybridization patterns from both inserts if the sequences are contiguous.

2. Chromosome walking technique. This protocol would be accomplished by using the insert from each clone to isolate the adjacent DNA fragment. For example, the DNA insert of clone 7 would be used as a probe to identify other recombinant plasmids in the DNA library that overlap with clone 7 DNA. These new recombinant plasmids will contain DNA sequences which extend on one side or the other from the fragment carried in clone 7. The direction of extension can be

determined by producing a restriction map of each fragment. This process must be repeated until reaching the overlap region of clone 2 DNA.

3. Isolation of the clones that contain inserts from both clones 2 and 7. This may be achieved by using the inserts from clones 2 and 7 to screen the DNA library by DNA hybridization. The clones that contain homologous sequences to both clones 2 and 7 inserts will be analyzed for their DNA inserts.

4. Two-dimensional gel electrophoresis. The possibility of different hemagglutinin components migrating the same distance in SDS-PAGE can be explored by analysis of *B. gingivalis* antigens in two dimensional gel electrophoresis and probing with the antisera against each clone.

5. Affinity purification of the polypeptide reactive with anti-clone 2 antiserum and determination of reactivity with anti-clone 7 antiserm. *B. gingivalis* antigens synthesized by clone 2 could be purified by immunoaffinity chromatography employing immobilized polyclonal antiserum against clone 2. The *Bacteroides* antigens which antiserum against clone 2 recognize will be bound to the column and will be eluted with a pH gradient. The isolated antigens could then be tested for reactivity with the antiserum against clone 7.

In addition, the isolated antigens should be tested for hemagglutination activity and analyzed in SDS-PAGE for determination of the molecular weight. The isolation of *B. gingivalis* antigens which possess hemagglutination activity should confirm that these cloned DNAs are hemagglutinin genes of *B. gingivalis*. The peptide maps or peptide fingerprints in two-dimensional gel electrophoresis of these isolated

proteins will definitely confirm that they are the same or different proteins and thus determine if one or more components of *B. gingivalis* mediate hemagglutination.

In order to define the genes which encode the hemagglutinin(s), subcloning should be performed. The DNA of functional subclones with the smallest insert could then be sequenced. The predicted amino acid sequence will provide information such as the isoelectric point (p^I) which would facilitate the purification of the protein.

Finally, the evidence which proves that hemagglutinin genes have been cloned must be generated by producing *B. gingivalis* mutants which lack hemagglutinating activity. If a transformation system is developed for *B. gingivalis*, it would then be possible to mutagenize the cloned sequence and introduce the mutated gene into *B. gingivalis*, thereby knocking out the hemagglutinating ability of the cells. *B. gingivalis* mutants devoid of hemagglutinating activity are necessary for *in vivo* studies to establish the significance of hemagglutination in pathogenesis. The cloning of hemagglutinin(s) which are suspected to mediate attachment to host tissues provides a means to define the native structures of *B. gingivalis* responsible for this activity. The cloned hemagglutinin genes are also good candidates for DNA probes for the rapid identification of *B. gingivalis* in clinical samples. Ultimately, these cloned genes may facilitate the production of a vaccine which prevents the colonization of *B. gingivalis* in the gingival sulcus and possibly the prevention of periodontal disease.

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BIOGRAPHICAL SKETCH

Somying was born to Sanguan and Chaufa Juijaitrong on December 2, 1953, in Tahmuang, Kanchanaburi, Thailand. Somying is married to Sornthep Tumwasorn and has 2 sons, Pattarawuth and Nattapol.

She lived in Tahmuang until 1969 when she went to Triem Udomsuksa School in Bangkok. She entered Kasetsart University in June, 1971 and was supported by the John F. Kennedy Foundation until she received a Bachelor of Science (with honours) in food science and technology in May, 1975. Upon graduation, she received a scholarship from the University Development Commission to persue a master's degree in microbiology at Kasetsart University. After graduation in May, 1977, she joined the Department of Microbiology, Faculty of Medicine, Chulalongkorn University as an instructor and was appointed as an Assistant Professor in 1980.

In August, 1983, Somying entered the Graduate School of the University of Florida in the Department of Immunology and Medical Microbiology. Her Ph.D. program has been arranged and supported in part by the Fulbright Foundation. She persued her dissertation research in the laboratory of Dr. Ann Progulske and was supported in part by the Division of Sponsored Research and a training grant from the National Institutes of Health. Somying plans to continue a molecular study of the pathogens which cause important infectious diseases in Thailand.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as dissertation for the degree of Doctor of Philosophy.

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